UNIVERSITY OF LONDON THESIS

Degree PhD Year 2007 Name of Author CHOWDHURY, Tamzina

Investigation of the role of MLL-ENL in leukaemogenesis

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A thesis submitted for the Degree of Doctor of Philosophy

2007

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Declaration

I, Tanzina Chowdhury, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Acute leukaemia is the most common form of childhood cancer and is the primary cause of cancer-related mortality in children. The major initiating event in infant leukaemia is rearrangement of the *Mll* gene at 11q23, and such leukaemias have a poor survival rate.

Chromosomal translocations occur commonly in leukaemia and an in-frame fusion gene is created with altered properties to the original genes involved. Translocations of chromosome 11q23 result in the fusion of *Mll* to a variety of partners genes. *Mll-Af9* results from t(9;11), *Mll-Af4* from t(4;11) and *Mll-Enl* from t(11;19) and are thought to cause aberrant transcriptional regulation.

To identify candidate target genes of t(11;19), MLL-ENL has been over-expressed in 32D cells, an immortalised myeloid progenitor cell line. The actions of MLL-ENL on cell survival following growth factor withdrawal, and on G-CSF mediated differentiation have been studied. Potential transcriptional targets of MLL-AF9 and MLL-ENL have been identified using microarray technology. Ten genes were identified as possible candidate target genes of the MLL-ENL protein that might be involved in leukaemogenesis. Of particular interest were *Gata1* and *HTm4* which have a role in haematopoiesis and cell cycle regulation. shRNA constructs were generated to knock down MLL-ENL and allow validation of the potential target genes.

The mechanism of leukaemic transformation by MLL-ENL has also been investigated. In leukaemia, fusion proteins may contribute to haematological malignancy in various ways, though the main routes are thought to be either a gain-of-function or dominant negative action. In order

to determine the mechanism by which a MLL fusion protein immortalizes murine haematopoietic progenitor cells (HPC), a system was developed where MLL-ENL was expressed in murine HPCs in the absence of both alleles of *Mll*. The experiments were carried out using murine HPCs from conditional *Mll* knockout mice generated in our laboratory where the wild-type *Mll* allele is flanked by LoxP sites at exons 9 and 10, and can be inactivated following Cre-mediated recombination. The ability of cells to undergo sequential re-plating in methylcellulose was used as an indication of immortalization. These experiments now definitively show that the MLL-ENL fusion protein acts in a gain-of-function manner.

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Acknowledgements

I would firstly like to thank my supervisor, Dr. Hugh Brady, for his supervision and support throughout my PhD, and I also remain exceptionally grateful to Dr. Owen Williams for his invaluable advice and guidance. I deeply thank my clinical supervisor, Professor Ian Hann, who was determined in driving a PhD programme for clinicians, and who has remained unfailing in his support and continuous encouragement. I also gratefully acknowledge Dr. Antony Michalski for his crucial advice to me over the last few months before submission. I continue to deeply appreciate the advice, support and friendship of Dr. Sarah Horton and of all my colleagues in the Department of Molecular Haematology and Cancer Biology.

I would particularly like to thank Dr. Jasper de Boer for his help in designing and conducting gene down-regulation experiments and for his constant advice and support, and I thank Kathryn McMahon without whom the analysis of aspects of her conditional knockout model would not have been possible. My particular thanks also to Nipurna Jina and Dr. Mike Hubank for their help in the design of Affymetrix microarray experiments, the running of the microarrays and help with data analysis. I am grateful to Jo Sinclair for her assistance with flow cytometry, and Ruth Lyons for advice regarding Q-PCR experiments and data analysis.

I express my deepest personal thanks particularly to Heathcliff whose love, concern and immense encouragement gave me focus and direction, to David Lyndon, who supported me with unparalleled selflessness and kindness throughout every aspect of my PhD, to Mike, the child under the tree at the very beginning who held my hand so tightly at the very end, and to Nic Wilson for constant, unwavering support and chocolate.

Above everything, I thank my parents, that I am blessed with their love, that I am sustained by it through every single day, and that I am filled with the gentle tenderness of their support in everything I do.

This thesis is dedicated to my Mother and Father
to whom I owe simply Everything

Abbreviations

2-ME	2-mercaptoethanol
ABL	acute biphenotypic leukaemia
AGM	aorta-gonad-mesonephros
ALL	acute lymphoblastic leukaemia
AML	acute myeloid leukaemia
APC	allophycocyanin
BCR	breakpoint cluster region
BM	bone marrow
cDNA	complementary DNA
CFU	colony forming-unit
CFU-GEMM	CFU-granulocyte-erythrocyte-macrophage-megakaryocyte
CFU-GM	CFU-granulocyte-macrophage
CFU-M	CFU-macrophage
cRNA	complementary RNA
CLP	common lymphoid progenitor
CML	chronic myeloid leukaemia
CMP	common myeloid progenitor
CMV	cytomegalovirus
CSF	colony stimulating factor
CSFR	colony stimulating factor receptor
CT	cycle threshold
DEPC	diethyl pyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dox	doxycycline
DTT	dithiothreitol

E13.5	embryonic day 13.5
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetra acetic acid
EGFP	enhanced green fluorescent protein
ES	embryonic stem
EST	expressed sequence tag
FACS	fluorescence activated cell sorting
FAM	6-carboxyfluorescein
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FL	foetal liver
G-CSF	granulocyte CSF
GM-CSF	granulocyte-macrophage CSF
GMP	granulocyte-monocyte progenitor
HA	haemagglutinin tag
HBSS	Hanks balanced saline solution
HDAC	histone deacetylase
HPC	haematopoietic progenitor cell
HRP	horse radish peroxidase
HSC	haematopoietic stem cell
IL	interleukin
IMDM	Iscove's modified Dulbecco medium
INT	p-iodonitrotetrazolium
IRES	internal ribosome entry site
IVT	in vitro transcription
kb	kilobases
kDa	kilo Daltons
LMPP	lymphoid primed multipotent progenitor
LSC	leukaemic stem cell
LT-HSC	long-term HSC
LTR	long terminal repeat

mAb	monoclonal antibody
MACS	magnetic activated cell sorting
MCS	multiple cloning site
MCSF	macrophage-CSF
MEM	minimal essential-apha medium
MEP	megakaryocyte-erythrocyte progenitor
MFI	mean fluorescence intensity
MGG	May Grünwald Giemsa
MMLV	Moloney murine leukaemia virus
MPP	multi-potent progenitor
mRNA	messenger RNA
MSCV	murine stem cell virus
MT	methyltransferase
NHEJ	non-homologous end joining
NK	natural killer
NOD/SCID	non-obese diabetic / severe combined immunodeficient
NP-40	nonidet P40
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PHD	plant homeodomain
PGK	phosphoglycerate kinase promoter
PVDF	polyvinylidene fluoride
Q-PCR	quantitative real-time PCR
RIPA	radioimmunoprecipitation assay
Rn	reaction value
RNA	ribonucleic acid
RT-PCR	reverse-transcription PCR
rtTA	reverse tetracycline transactivator
SCF	stem cell factor
SIN	self-inactivating

SDS	sodium dodecyl sulphate
SNL	sub-nuclear localisation
ST-HSC	short-term HSC
ST-LSC	short-term LSC
SV40	simian virus 40
t-AML	therapy related AML
TAMRA	6-carboxy-tetramethylrhodamine
TE	Tris-EDTA
TetR	tetracycline repressor
topo II	topoisomerase II
TRE	tetracycline response element
tTA	tetracycline transactivator
tTS	tetracycline suppressor
UNG	uracil-N-glycosylase
VP16	virion protein 16

1 Introduction

1.1 Haematopoiesis

Understanding the general aspects of normal blood cell formation is important when considering the ways in which a leukaemic cell may arise. Haematopoiesis is a highly ordered and complex process by which all mature blood cells are generated from a small subset of pluripotent haematopoietic stem cells (HSC). These mature blood cells consist of erythrocytes and platelets, leukocytes comprising granulocytes and monocytes, and lymphocytes. Developmental control of the different blood cell lineages involves progenitor cell exposure to haematopoietic cytokines or growth regulators, as well as the regulated expression of genes by transcription factors (Engel and Murre 1999).

The initial sites of haematopoiesis within mammalian embryos are the yolk sac blood island, para-aortic splanchnopleura (P-Sp), and aorto-gonado-mesonephros region (AGM) (Medvinsky and Dzierzak 1996, Zon 1995). In the mouse embryo where this process has been most extensively studied, primitive haematopoiesis begins on embryonic day 7 (E7) with a burst of primitive nucleated erythrocytes that arise from the bi-potential haemangioblast which is capable of forming embryonic blood cells as well as blood vessels from endothelial cells (Moore and Metcalf 1970). Primitive haematopoiesis continues in the P-Sp from E7.5 to E9.5, and then in the AGM from E10.5-E11.5. HSC capable of long term repopulation of the entire adult haematopoietic system are generated at this stage but require a subsequent maturation step within the foetal liver which remains the main organ of definitive haematopoiesis, until bone marrow engraftment can be achieved and definitive haematopoiesis continues after birth (Baron and Fraser 2005, Keller, *et al* 1999).

Of importance to normal development and theories regarding leukaemogenesis is the capacity of stem cells to self-renew and regulate the relative balance between self-renewal and differentiation. Mouse multipotent progenitors can be divided into long-term (LT-) and short-term (ST-) HSC with the potential to renew, as well as multipotent progenitors (MPP) without detectable self-renewal potential (Spangrude, *et al* 1988). The classical model for haematopoietic commitment and blood lineage development is shown schematically in Figure 1.1A. In this model the first and decisive lineage commitment step occurs in LT-HSC. This results in the generation of daughter cells with increased lineage commitment, and more limited ability to self-renew, termed ST-HSC (Akashi, *et al* 2000, Reya, *et al* 2001, Shizuru, *et al* 2005). The ST-HSC gives rise to the MPP which gives rise to either the common lymphoid or common myeloid progenitors (CLP and CMP respectively), with the capacities for differentiation into more limited cell types (Akashi, *et al* 2000, Shizuru, *et al* 2005). The CLP is capable of generating natural killer (NK) cells which lack typical immunophenotypic markers of T- or B-cells and are capable of lysing a variety of tumour or virus infected cells without any obvious antigenic stimulation (Kondo, *et al* 1997). The CLP also gives rise to both T and B lymphocyte progenitors (pro-T and pro-B cells respectively) with subsequent generation of T and B lymphocytes. The CMP give rise to granulocyte-macrophage- progenitors (GMP) and megakaryocyte-erythroid progenitors (MEP). The GMP gives rise to mature granulocytes (neutrophils, eosinophils and basophils), and monocytes, while mature erythrocytes and platelet-producing megakaryocytes are derived from the MEP (Akashi, *et al* 2000).

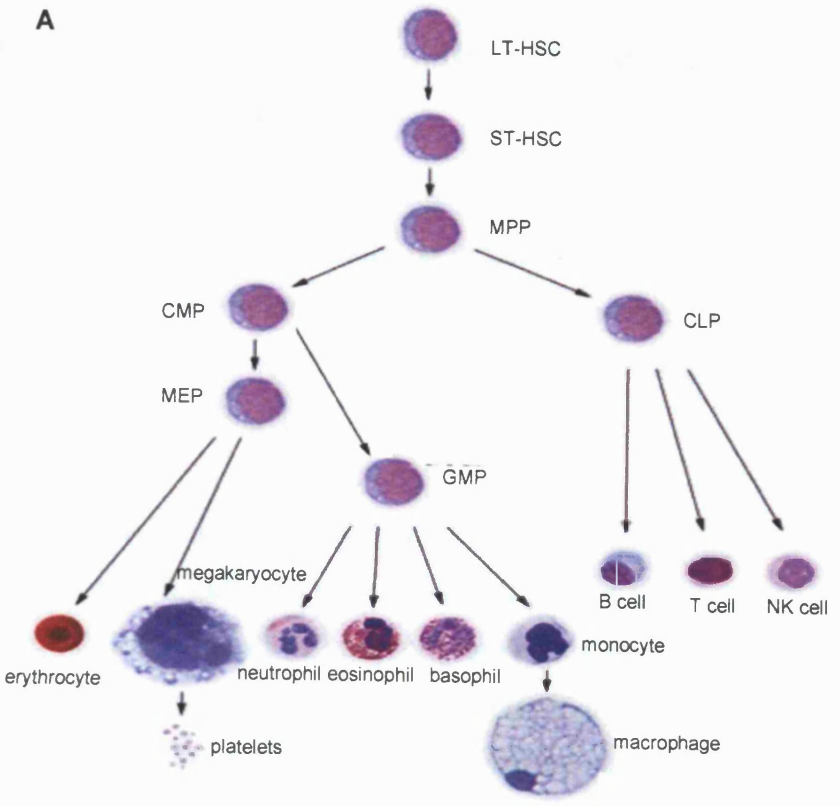
More recently this classical model of haematopoietic commitment has been challenged in the light of further investigation into the molecular pathways involved. ST-HSC which co-expressed high levels of the FMS-like tyrosine kinase 3 (FLT3) receptor appeared to sustain granulocyte, monocyte, and

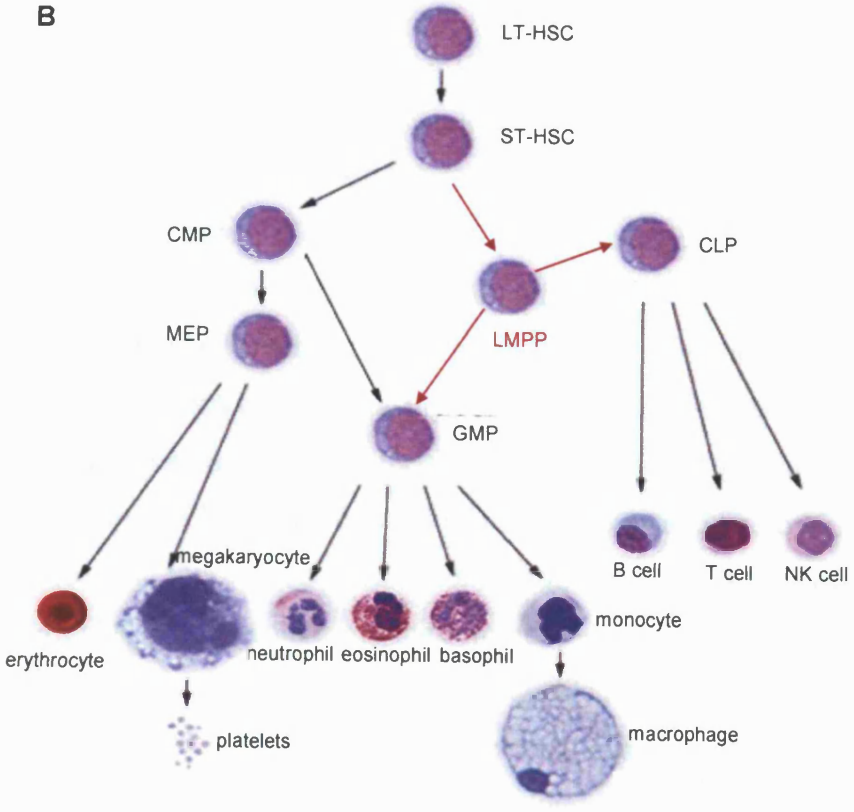
lymphocyte potentials, but were restricted in their ability to produce significant erythroid and megakaryocyte progeny (Adolfsson, *et al* 2005). The alternative model proposed is that the ST-HSC develops into a lymphoid primed multipotent progenitor (LMPP) following the loss of its megakaryocyte and erythroid potential. Cells retaining this potential derived from the ST-HSC are termed MEP. The LMPP then generates a CLP following loss of its GM potential. Cells retaining GM potential are derived from the LMPP and are termed GMP. Caveats of this proposed model, however, are that there needs to be a demonstration that LMPP do give rise to GMP, and that it is yet to be determined whether the proportion of GMP arising through the alternative pathway is similar to that arising from the classical route as implied by the model (Hock and Orkin 2005). This proposed model of haematopoietic lineage commitment is incorporated into the classical model by the authors and a composite model is shown in Figure 1.1B, which suggests that GMP are derived from either the LMPP or the CMP, and that both the latter two cell types co-exist.

Figure 1.1 Pathways of haematopoietic cell commitment.

A shows the traditional model of haematopoiesis in which long-term haematopoietic stem cells (LT-HSC) give rise to short-term haematopoietic stem cells (ST-HSC) with a more limited self-renewal capacity. ST-HSC give rise to multipotent progenitors (MPP) which give rise to the common myeloid progenitor (CMP) and common lymphoid progenitor (CLP). Myeloid and lymphoid mature cell types respectively are derived from these progenitors. MEP: megakaryocyte-erythrocyte progenitor, GMP: granulocyte-macrophage progenitor.

B shows a composite model (Adolfsson, *et al* 2005) in which ST-HSC give rise to the lymphoid primed multi-potent progenitor (LMPP) and CMP. The LMPP gives rise to both the CLP and GMP. Mature cells arise from the MEP, GMP and CLP as previously shown.


A
LT-HSC
ST-HSC
MPP
CMP
CLP
MEP
GMP
megakaryocyte
B cell
T cell
NK cell
erythrocyte
neutrophil
eosinophil
basophil
monocyte
platelets
macrophage


B
LT-HSC
ST-HSC
CMP
CLP
LMPP
MEP
GMP
megakaryocyte
B cell
T cell
NK cell
erythrocyte
neutrophil
eosinophil
basophil
monocyte
platelets
macrophage

1.2 Transcriptional regulation of haematopoietic development

The ability to manipulate the mouse genome via homologous recombination into embryonic stem (ES) cells has defined the role of a number of genes in the transcriptional control of haematopoiesis, particularly in determining self-renewal ability of HSC and lineage commitment (reviewed in (Baron and Fraser 2005, Engel and Murre 1999).

The haemangioblast is the common progenitor that gives rise to primitive haematopoietic cells and endothelial cells, and requires expression of the stem cell leukaemia (SCL) transcription factor in addition to the vascular endothelial growth factor (VEGF) receptor if angioblast fate is not to occur (Ema, *et al* 2003). Development and localization of embryonic HSC appears to involve different factors to those which govern the function of mature HSC in the adult. Primitive haematopoiesis results in the production of transient large nucleated erythroblasts, megakaryocytes and immature macrophages, and is stimulated by VEGF signalling. SCL and LIM domain only 2 (LMO2) are essential transcriptional regulators of early haematopoiesis (Robb, *et al* 1995, Warren, *et al* 1994), while globin transcription factor 1 (GATA1) and GATA2 functions overlap (Fujiwara, *et al* 2004) . Transcriptional regulation of definitive haematopoiesis requires acute myeloid leukaemia 1 (AML1), subunit b of core binding factor (CBF/B), and expression of the myeloblastosis oncogene *cmyb* which, if mutated, cause widespread defects in definitive haematopoiesis but do not affect primitive erythropoiesis (Okuda, *et al* 1996), and loss of GATA2 expression at this point cannot be compensated by GATA1 (Fujiwara, *et al* 2004). However, in contrast to the continued expression of other factors required to determine and maintain lineage identity and function, expression of SCL in HSC and MPP does not contribute to HSC engraftment, self-renewal and differentiation into myeloid and lymphoid

lineages (Mikkola, *et al* 2003b). A related transcription factor, lymphoblastomic leukaemia 1 (LYL1), however, is required for normal HSC function as well as B-cell differentiation, and *Lyl1* null fetal liver and bone marrow cells fail in their competitive reconstituting abilities (Capron, *et al* 2006). Interestingly, *Scl* and *Lyl1* are regulated by the same E26 avian leukaemia oncogene (ETS) and GATA factors, and may explain the mild phenotype of *Scl* null HSC when *Lyl1* expression is intact (Chan, *et al* 2007).

The development of distinct lineages from MPP is controlled by cell-restricted transcription factors and exposure to haematopoietic cytokines, and deregulation of normal transcription is implicated in the development of leukaemias associated with chromosomal translocations (Rabbitts, *et al* 1999). Gene targeting studies and enforced expression in cells capable of differentiation have allowed the identification of factors that essentially determine or influence lineage commitment from MPP.

1.2.1 Transcription factors involved in myeloid and lymphoid lineage determination

As well as being required for the definitive development of all haematopoietic lineages, AML1 and CBF/B are required for the normal differentiation and proliferation of myeloid and lymphoid cells. Both genes are commonly rearranged in t(8;21) and inversion (16) acute myeloid leukaemia (AML), and in t(12;21) common acute lymphoblastic leukaemia (ALL) (Lo Coco, *et al* 1997, McHale and Smith 2004). PU.1 belongs to the ETS-family of transcriptional regulators and determines HSC self-renewal as well directing lineage development by binding to the promoters of many myeloid genes including the macrophage colony-stimulating factor (M-CSF) receptor, granulocyte-macrophage (GM)-CSF receptor alpha, and the alpha sub-unit CD11b of the integrin Mac1 (Dakic, *et al* 2005, Gangenahalli, *et al* 2005, Iwasaki, *et al* 2005). In B cells, it regulates the

immunoglobulin lambda 2-4 and kappa 3' enhancers, and J chain promoters (reviewed in (Gangenahalli, *et al* 2005).

GATA1 and friend of Gata1 (FOG1) are essential transcription factors in erythroid and megakaryocytic lineages (Orkin 1992, Shivdasani, *et al* 1997), and both GATA1 and GATA2 may interact with PU.1 to inhibit its function. Overexpression of GATA1 in early myeloid cells blocks myeloid development (Visvader, *et al* 1992). Pathway inhibition also results in myeloid development, and over expression of PU.1 can block erythroid development in proerythroblasts and result in erythroblast immortalisation and in erythroleukaemia in mice (Moreau-Gachelin, *et al* 1996). This suggests an ability of a particular transcription factor to upregulate lineage-specific target genes in a cell capable of differentiation into more than one lineage, depending on the degree of expression of that particular transcription factor compared to another. In a model of myeloid development by PU.1, both PU.1 and GATA1 are expressed at low levels in CMP and specific upregulation of PU.1 is associated with a decrease in GATA1 expression due to direct binding of GATA1 by PU.1. Activation of specific genes by PU.1 results in increased proliferation, differentiation and suppression of apoptosis of myeloid progenitors in a programme of myeloid lineage commitment (Zhang, *et al* 1999, Zhang, *et al* 2000). The amplification of specific transcriptional programmes associated with a specific lineage, with associated repression of those of other lineages, in the presence of specific transcription factors, is likely to be the mechanism by which MPP become committed to specific lineages.

CCAAT/enhancer binding proteins (C/EBP) are upregulated during myelopoiesis and C/EBPe is a regulator of terminal differentiation of eosinophils and functional maturation of neutrophils (reviewed in (Yamanaka, *et al* 1998). C/EBPa protein is present at high levels in the myeloblastic murine 32Dcl3 cell line which is used in this study, and this

protein is regulated during differentiation by G-CSF. Upon G-CSF treatment the levels of C/EBPa increase 2-fold within 24 hours, and are maintained for several days before falling to low levels by day 6 of differentiation (Scott, *et al* 1992). G-CSF treatment is also used in this study to examine effects of oncogene expression on differentiation.

B cell development involves lineage determination through co-ordinated transcriptional regulation, together with pre-B cell receptor selection, and subsequent B cell tolerance during the immature-to-mature B cell transition (reviewed in (Busslinger 2004, Hayakawa, *et al* 1997, Schebesta, *et al* 2002). Illustrative examples of transcription factors involved in B cell development are described below.

Gene targeting studies show that the initiation of B-cell development in the bone marrow depends on the B-cell specific, basic helix-loop-helix transcription factor E2A and early B-cell factor EBF (Zhuang, *et al* 2004, Zhuang, *et al* 1994). These proteins regulate B-cell specific activator protein (BSAP), the transcription factor encoded by the *Pax5* gene, which is critical to B-cell development in suppressing alternative lineage cell fates early in development, and *Pax5* null pro-B cells can generate both myeloid and T lineage cells. Studies using a *Pax5* conditional knockout mouse has revealed that PAX5 expression must be maintained through the pro-B stage, as loss of PAX5 results in committed pro-B cells reverting to multi-lineage potential cells (Nutt, *et al* 1999, Rolink, *et al* 1999). The t(17;19) translocation results in the formation of the *E2a*-hepatic leukaemic factor (*Hlf*) fusion gene in which the expression of HLF is aberrantly driven by the *E2a* promoter, implicating the deregulation of these genes in a rare childhood pro-B ALL (reviewed in (Seidel and Look 2001).

1.2.2 The structure and function of the MLL gene in normal haematopoiesis

The mixed lineage leukaemia (*Mll)* gene spans 90 kb of chromosome 11q23, has 38 exons (Nilson, *et al* 1996), and codes for a protein of 3969 amino acids (Tkachuk, *et al* 1992). The *Mll* gene and its protein product are shown in Figure 1.2.

The MLL protein product shows structural similarities to Drosophila Trithorax (TRX) protein, with strong homology shared in the SET (Su(var)3-9, enhancer-of-zest) and PHD (plant homeodomain) domains (Tkachuk, *et al* 1992), and these domains are associated with a role in transcriptional regulation by modification of chromatin (Fair, *et al* 2001). The PHD domain mediates transcriptional repression through its binding of peptidyl-prolyl isomerase E (Cyp33), and other well-characterised domains are also implicated in the regulation of chromatin structure and transcription.

Three AT hook domains located in the amino terminus of MLL bind DNA that has AT-rich regions with a particular cruciform or bent tertiary structure rather than doing so in a sequence specific manner (Aravind and Landsman 1998). Two nuclear localisation domains in the amino terminal region confer a punctate nuclear expression pattern on the MLL protein (Yano, *et al* 1997). The DNA methyltransferase homology domain (MT) provides DNA binding ability in addition to the AT hook domains, enabling MLL to bind unmethylated CpG (cytidine and guanine separated by phosphate) sequences with high specificity (Birke, *et al* 2002) and direct methylation of these sequences would normally result in transcriptional silencing. Transcription is repressed during interaction of the DNA methyltransferase domain with an active promoter in reporter assays and thought to be mediated by interaction with histone deacetylases or the polycomb group proteins histone periodic control 2 (HPC2) and murine

leukaemia viral oncogene homologue (BMI1) (Prasad, *et al* 1995, Xia, *et al* 2003, Zeleznik-Le, *et al* 1994) and may involve chromatin modification by MLL. A transactivation domain (TAD) is located between the PHD and SET domains (Prasad, *et al* 1995) and facilitates the binding of MLL to the cAMP binding protein (CBP) to enhance transcriptional activation by promoting its binding with cAMP response element binding (CREB) (Ernst, *et al* 2001).

The MLL protein has a predicted molecular weight of 430kDa, though actually migrates at ~500kDa, and is proteolytically processed by Taspase1 *in vivo* at a conserved site between the PHD and transactivation domains to generate two polypeptides of 180 and 320 kDa (Hsieh, *et al* 2003), where the N-terminal polypeptide retains transcriptional repression properties and the C-terminal domain contains transcriptional activation properties. Although the cleavage of MLL separates the transrepression and transactivation domains of MLL, immunoprecipitation of either polypeptide isolates almost identical protein complexes, suggesting MLL exists as a heterodimer in part of a single complex, and not as two separate complexes (Hsieh, *et al* 2003, Nakamura, *et al* 2002, Yokoyama, *et al* 2002). A mouse knockout model of *Taspase 1* provides evidence that dimerisation of MLL is important for normal MLL function, and the majority of *Taspase 1*$^{-/-}$ mice die at approximately 2 days post-partum (Takeda, *et al* 2006). Full length MLL is still present in murine embryonic fibroblasts but the cleaved MLL protein cannot be detected. The lethality seen in this model is not similar to the early embryonic death seen in *Mll*$^{-/-}$ mice ((Yagi, *et al* 1998, Yu, *et al* 1995), and suggests that the residual full length MLL has some normal function that maintains the mice to a later stage of survival (Takeda, *et al* 2006).

MLL is now known to be part of a super-complex containing proteins associated with other complexes such as SWI/SNF, NuRD, Sin3A, TFIID

and the yeast SET1 homologues as well as the hSNF2H protein, which acetylate, deacetylate and methylate histones thus regulating gene transcription (Nakamura, *et al* 2002). The SWI/SNF complex and hSNF2H are involved in chromatin remodelling, the NuRD complex has histone deacetylase and nucleosome remodelling activity, the Sin3A complex has a histone deacetylase function, and the TFIID complex acts in transcriptional activation (Lemon, *et al* 2001).

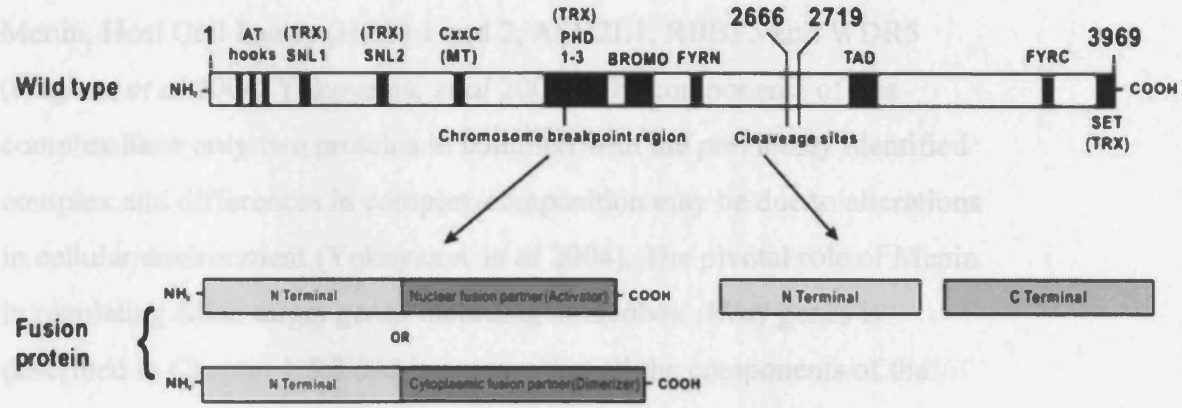


Figure 1.2 Structure of the wild-type MLL protein and schematic view of an MLL fusion protein.

The 430 kDa MLL protein is proteolytically processed by Taspase1 *in vivo* at a conserved cleavage site between the PHD and transactivation domains to generate two polypeptides of 180 and 300 kDa, where the N-terminal polypeptide retains transcriptional repression properties and the C-terminal domain contains transcriptional activation properties. These fragments interact *in vivo* via the FYRN domain of p300 and the FYRC domain of p180. Cleavage at the breakpoint region and fusion of the N-terminal fragment with a nuclear or cytoplasmic partner protein results in an aberrant fusion protein.

AT hooks: AT hook DNA binding motifs, SNL 1 and 2: speckled nuclear localization signals 1 and 2, CxxC (MT): cysteine-rich motif homologous to DNA methyltransferase and MBD1, PHD 1−3: PHD fingers 1, 2 and 3, BROMO: BROMO domain, FYRN: F/Y-rich region, N-terminus, FYRC: F/Y-rich region, C-terminus, TAD: transactivation domain, SET: SET domain. Regions structurally conserved with Trithorax of *Drosophila* are indicated by the (TRX) label. Reproduced from (Li, *et al* 2005).

A further complex has been identified in which MLL is contained with Menin, Host Cell Factor (HCF) 1 and 2, ASH2L1, RBBP5 and WDR5 (Hughes, *et al* 2004, Yokoyama, *et al* 2004). The components of this complex have only two proteins in common with the previously identified complex and differences in complex composition may be due to alterations in cellular environment (Yokoyama, *et al* 2004). The pivotal role of Menin in regulating MLL target genes including homeobox *(Hox)* genes is described in Chapter 1.5.3 and it appears that all the components of the complex bind to the promoter of *Hoxa9* only when MLL is present (Nakamura, *et al* 2002).The role of the proteins within the supercomplex appear to have a significant role in the transcriptional activation properties of MLL through a chromatin remodeling function which may have implications for the way in which MLL fusion proteins function in human leukaemia.

MLL is expressed in haematopoietic and a variety of non-haematopoietic tissues (Butler, *et al* 1997). The normal function of MLL has been examined by gene targeting studies which show striking abnormalities of haematopoiesis and a number of defects in non-haematopoietic tissues in MLL knockout mice (Yagi, *et al* 1998, Yu, *et al* 1998, Yu, *et al* 1995). Both haematopoietic and non-haematopoietic defects in MLL knockout models appear to be due to aberrant regulation of *Hox* genes by MLL. *Hox* genes are key regulators of haematopoiesis as well as of the mammalian body plan, determining the axial skeleton, hind brain and limb development during embryogenesis (Boncinelli, *et al* 1989, Grier, *et al* 2005, Lawrence and Largman 1992).

Mll $^{-/-}$ mice die between E10.5 and 14.5 (Hess, *et al* 1997, Yagi, *et al* 1998, Yu, *et al* 1998, Yu, *et al* 1995). These mice show axial skeletal abnormalities, anaemia and thrombocytopoenia and a marked decrease in

the number of haematopoietic cell precursors in the yolk sac, although all cell types are present (Hess, *et al* 1997, Yu, *et al* 1995).

Investigation into the role of MLL in definitive haematopoiesis has previously shown that *Mll* $^{-/-}$ ES cells are unable to contribute to the foetal liver haematopoietic stem cell population in chimaeric mice (Ernst, *et al* 2004a). Cells expressing the receptor tyrosine kinase cKIT that were present in the AGM of *Mll* $^{-/-}$ mice did not have normal expression of HSC cell surface marker, and cells derived from the E11.5 AGM region of *Mll* $^{-/-}$ mice were unable to reconstitute recombinant activating 2 (*Rag*-2$^{-/-}$) mice, while those derived from *Mll* $^{+/-}$ mice were unable to generate HSC at normal frequencies (Ernst, *et al* 2004a). These studies suggested that MLL is required for primitive haematopoietic cells to generate normally functioning HSC, either directly or by promoting their ability to undergo maturation in the fetal liver. However, the absolute requirement for MLL has been challenged more recently by studies which show that grossly normal haematopoiesis involving all cell lineages is observed in adult mice in which *Mll* is deleted in haematopoietic cells only (McMahon, *et al* 2007 manuscript submitted). However, in competitive repopulation assays, *Mll* deficient bone marrow cells were unable to compete with wild type cells, again suggesting that *Mll* is needed for the correct development of foetal liver haematopoiesis and also to retain self-renewal potential in adult haematopoietic cells (McMahon, *et al* 2007 manuscript submitted).

The role of MLL in driving proliferation of haematopoietic progenitors is shown by the reduced ability of *Mll*-deficient yolk sac and fetal liver cells to establish normal colony formation in vitro (Hess, *et al* 1997, Yagi, *et al* 1998). A reduction in the size of all colony forming units was observed, while the cell phenotype within each unit was preserved and identical to those from *Mll* $^{+/-}$ or *Mll* $^{-/-}$ embryos. Thus, MLL does not appear to be required for late-stage differentiation of these progenitors. However,

although *Mll* $^{-/-}$ ES cells were able to generate normal embryoid bodies to give rise to a haemangioblast with the ability to commit to both haematopoietic and endothelial cells, MLL was required for the early differentiation and proliferation of haematopoietic cells derived from *Mll* $^{-/-}$ mesoderm, and such haematopoietic cells failed to form haematopoietic colonies in vitro (Ernst, *et al* 2004a, Ernst, *et al* 2004b).

Analysis of *Hox* gene expression identifies the role of MLL in maintaining, but not initiating the expression of these genes. *Hoxa7* and *Hoxc8* genes are initially expressed normally but not sustained (Yu, *et al* 1998), and MLL has also been shown to maintain the expression of *Hoxa9, Hoxa10, Hoxb4, Hoxb5, Hoxb6, Hoxb8, Hoxc6* and *Hoxc9* (Ernst, *et al* 2004b, Hanson, *et al* 1999). Heterozygous mutant embryos show a one-segment posterior shift in *Hoxc8* expression (Hanson, *et al* 1999). The actions of Polycomb group proteins, such as BMI1 which also has a SET domain, antagonise Trithorax group proteins, and alter chromatin structure to repress transcription of target genes (Kennison 1995, Orlando 2003, Schumacher and Magnuson 1997). In contrast to *Mll* mutants, embryos deficient for *Bmi1* show a one-segment anterior shift in *Hoxc8* expression and some of the defects associated with *Mll* $^{-/-}$ mice can be reversed by loss of *Bmi1* gene expression (Hanson, *et al* 1999). These studies propose a definitive role for MLL in normal haematopoiesis through its regulation of *Hox* genes, which in turn are essential to this process.

1.2.3 The role of *Hox* genes in regulating haematopoiesis

Hox genes are critical to normal haematopoietic development and are maintained by normal MLL protein expression, such that failure to express a subset of *Hox* genes may underlie the haematopoietic defect in *Mll* knockout mice (Yagi, *et al* 1998, Yu, *et al* 1995). Aberrant *Hox* gene expression is seen in microarray studies of over-expressed MLL-fusion proteins in vitro (Antonchuk, *et al* 2001, Armstrong, *et al* 2002, Debernardi,

et al 2003, Tsutsumi, *et al* 2003, Yeoh, *et al* 2002), and is associated with the development of leukaemia in mouse models of MLL fusion leukaemias, and their importance as potential targets of MLL fusion proteins in causing leukaemia is investigated as part of this study. An understanding of the role of *Hox* gene expression in normal haematopoiesis and its relation to normal MLL function may inform an understanding of the way in which aberrant *Hox* gene expression may result from MLL-fusion expression and contribute to leukaemia.

Homeotic mutations in *Drosophila* result in one body segment being transformed into another, and give rise to the term *Homeobox.* This conserved 180 nucleotide region shared by the genes involved in these mutations encodes a 60 amino acid DNA-binding motif, and forms a helix-turn-helix structure, known as the homeodomain (Gehring, *et al* 1994). The development of the *Drosophila* embryo is guided by the precise temporal and spatial pattern of expression of these homeobox genes. The homeotic cluster of homeobox genes (HOM-C) is expressed in ordered domains, from 3' to 5', to parallel the anterior to posterior development of the axial body plan. Mammalian homeobox-containing genes that are expressed in haematopoietic cells include the hexapeptide group including the *Hox* genes containing an N-terminal 6-amino acid protein interaction domain, the POU group containing an N-terminal POU domain, the LIM group with an N-terminal LIM domain in addition to the homeodomain, and the TALE group including PBX and Meis proteins with homeodomains containing three extra amino acids. Homeobox proteins bind to DNA with increased affinity in combination with partner proteins such as PBX.

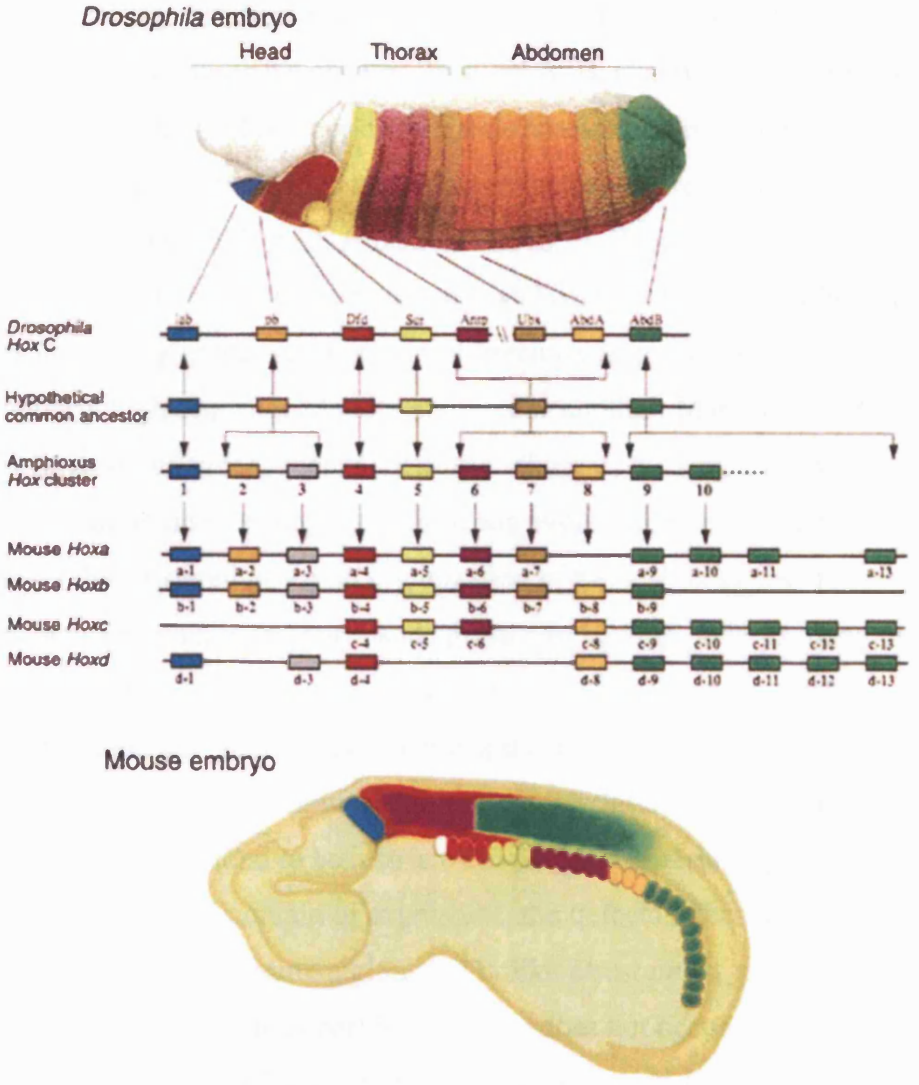


Figure 1.3 The genomic organisation of mammalian *Hox* genes.
Four conserved clusters, labelled A, B, C and D, containing 9 to 11 genes are organized on four different chromosomes, and each gene is numbered according to its 3' to 5' order on the chromosome. The thirteen paralog groups have a particularly high sequence identity among one another. The genes are expressed in temporal and spatial overlapping patterns along the body axes during murine embryogenesis to specify axial spine, limb, gastrointestinal and genitourinary development, and are expressed in a 3' to 5' direction. Reproduced from (Carroll 2000).

The genomic organization of the 39 mammalian *Hox* genes is shown in Figure 1.3. Four conserved clusters, labelled A, B, C and D, containing 9 to 11 genes are organized on four different chromosomes (Boncinelli, *et al* 1989). Each gene is numbered according to its 3' to 5' order on the chromosome, such as *Hoxa1, Hoxb2* and so forth. Genes that are in the same position on different clusters, such as *Hoxa4, Hoxb4, Hoxc4* belong to the same paralog group, and there are thirteen paralog groups, and have a particularly high sequence identity among one another. Mammalian *Hox* genes have significant sequence identity to those of the single HOM-C cluster found in insects, suggesting a strong evolutionary pressure to maintain the clustered genomic organization of homeobox genes. These genes are expressed in temporal and spatial overlapping patterns along the body axes during murine embryogenesis to specify axial spine, limb, gastrointestinal and genitourinary development, and are expressed in a 3' to 5' direction. *Hox*$^{-/-}$ mice show multiple developmental abnormalities, and these abnormalities tend to be restricted to the most anterior region of the deleted gene's usual domain of expression, the defects affect several different tissues in the same region, and unlike *Drosophila*, true transformation of one body part into another does not occur, but rather a loss of structures (Boncinelli, *et al* 1989, Lawrence and Largman 1992).

The normal stem cell and committed progenitor compartment of normal bone marrow expresses multiple genes of the *HOXA, HOXB and HOXC* clusters (Giampaolo, *et al* 1994, Moretti, *et al* 1994, Sauvageau, *et al* 1994), and *Hox* gene expression is highest in the most primitive subpopulation (Sauvageau, *et al* 1994). Genes that are located at the 3' region of the clusters are down regulated at the committed progenitor stage, while those at the 5' end are maintained until cells are undergoing the later stages of differentiation (Kawagoe 1999). Such genes include *Hoxa9, Hoxb9,* and *Hoxa10* (Sauvageau, *et al* 1994) whose aberrant expression is implicated in leukaemogenesis described below.

Murine models suggest that *HOXA, HOXB, and HOXC* cluster genes are expressed in early mouse yolk sac at the same time as the onset of haematopoiesis (McGrath and Palis 1997). Loss-of-function studies of *Hoxa9* show that haematopoiesis is affected at the level of the stem cell as well as the committed progenitor. *Hoxa9* deficient mice were viable and fertile with only mild skeletal defects, but defective haematopoiesis of T and B cells and granulocytes occurred (Lawrence, *et al* 1997). In vivo assays suggested that *Hoxa9* knockout mice had defects in their HSC with a ten-fold greater defect in ability to reconstitute the bone marrow compartment of sublethally-irradiated syngeneic mice compared to wild-type bone marrow (Lawrence, *et al* 1997).

Failure to down-regulate 3' *Hox* genes appears to result in a failure of cell differentiation and causes cell transformation. Retroviral transduction experiments to over-express specific *Hox* genes showed that *Hoxa10* over-expression was associated with large numbers of megakaryocyte/blast progenitors which evolved into AML (Thorsteinsdottir, *et al* 2002, Thorsteinsdottir, *et al* 1997). A marked reduction in pre-B-cell progenitors was also observed as well as a loss of committed macrophage progenitors suggesting that down-regulation of *Hoxa10* is required for B cell development and myeloid differentiation (Thorsteinsdottir, *et al* 2002, Thorsteinsdottir, *et al* 1997). Similarly, over-expression of *Hoxa9* also results in the development of AML following an expansion of HSC and myeloid precursors (Kroon, *et al* 1998, Thorsteinsdottir, *et al* 2002), and again a role for *Hoxa9* in blocking HPC differentiation is suggested. Retroviral over-expression of *Hoxb3* blocks both B- and T-cell development and causes a myeloproliferative disorder that progresses to AML (Sauvageau, *et al* 1995). In contrast, over-expression of *Hoxb4*, resulted in only mild elevations in the myeloid and B-cell compartments with no effect on cell differentiation and no development of leukaemia, but

did appear to positively regulate the expansion of the HSC pool (Antonchuk, *et al* 2001, Sauvageau, *et al* 1995, Thorsteinsdottir, *et al* 1999). *HoxA, HoxB* and *HoxC* gene expression is reduced in the deficient number of haematopoietic cells derived from embryoid bodies lacking *Mll*, and these cells are unable to divide and differentiate normally to form colonies. The ability of cells to differentiate, is restored by re-expression of *Hoxa9, Hoxa10* and *Hoxb4* genes with subsequent colony phenotype depending on the individual gene re-expressed (Ernst, *et al* 2004b). This suggests that individual *Hox* genes are able to rescue the ability of cells to differentiate in the absence of *Mll,* but are redundant in terms of conferring a proliferative function upon these cells.

1.3 Chromosomal translocations in paediatric acute leukaemia

The main subtypes of ALL and AML in childhood involve a variety of genetic alterations and are characterised by gross chromosomal changes which may be numerical, or structural in the form of translocations. Chromosomal translocations involve illegitimate recombination events or the juxtaposition of normally separated genes. Such a process can result in the deregulation of an oncogene by association with a powerful active regulatory element, as in the case of the *cMYC* oncogene juxtaposed with the immunoglobulin heavy chain enhancer.

Alternatively, and more commonly in leukaemogenesis, an in-frame fusion gene is created with altered properties to the original genes involved, and which usually has activated kinase activity or properties of novel transcriptional regulation (Greaves 2003, Greaves and Wiemels 2003). Such fusion genes are formed as a result of a number of chromosomal translocations in paediatric leukaemia, which include translocations of chromosome 11q23 resulting in the fusion of *MLL* to one of up to 50 known

partners genes. These fusion genes are associated with approximately 80% of infant leukaemias and confer a very poor survival in ALL cases (Pui, *et al* 2002), and are also associated with the majority of therapy-related leukaemias (Pui and Relling 2000). The fusion genes *MLL-AF4, MLL-ENL* and *MLL-AF9* are the three most frequently found in childhood leukaemias. *MLL-AF4* fusions are almost exclusively associated with ALL, *MLL-ENL* found in both AML and ALL cases, while *MLL-AF9* is mainly associated with AML, reflecting a tumour tropism which may allow clues to the biology of these leukaemias.

The main repair mechanism underlying aberrant recombination is thought to be non-homologous end-joining (Greaves and Wiemels 2003). In addition, it is known that inhibitors of topoisomerase II interfere with the re-ligation of DNA double strand breaks induced during chromosomal replication by topoisomerase II, thus exposing DNA free ends to allow translocations to occur. Most therapy-related leukaemias are *MLL*-rearranged leukaemias which have developed following previous treatment with topoisomerase II inhibitors for earlier malignancies. This, together with an association of maternal exposure to naturally occurring topoisomerase II inhibitors and *MLL*-rearranged infant leukaemias suggests that 11q23 translocations occur *in utero* as a result of interference with DNA repair (Alexander, *et al* 2001).

Other fusion genes arise from translocations associated with paediatric leukaemia. These are shown in Table 1.

Table 1 Chromosomal translocations and respective fusion genes found in paediatric leukaemia.

Disease	Translocation	Fusion protein
ALL	t(9;22)(q34;q11)	BCR-ABL
	(12;21)(p13;q22)	TEL-AML
	t(1;19)(q23;p13)	E2A-PBX1
	t(17;19)(q22;p13)	E2A-HLF
AML	inv (16)	CBFβ-MYH11
	t(8;21)(q22;q22)	AML-1-ETO
	t(15;17)(q22;q11.2)	PML-RARα

1.4 Neoplastic transformation and leukaemogenesis

1.4.1 Evolution of the malignant clone

The malignant transformation of cells to cause cancer is thought to follow a model in which there is the sequential accumulation of multiple genetic aberrations in a cell. This initially causes a limited expansion of the progeny of a single cell, and subsequent mutations result in new clonal outgrowth with a deregulated program of differentiation, proliferation or survival (Cavenee and White 1995, Greaves and Wiemels 2003). This model is illustrated in Figure 1.4. Only some of the acquired genetic aberrations in the final tumour will be critical to the transforming process, and identifying these initiating events will increase an understanding of disease biology and opportunities to develop targeted therapies.

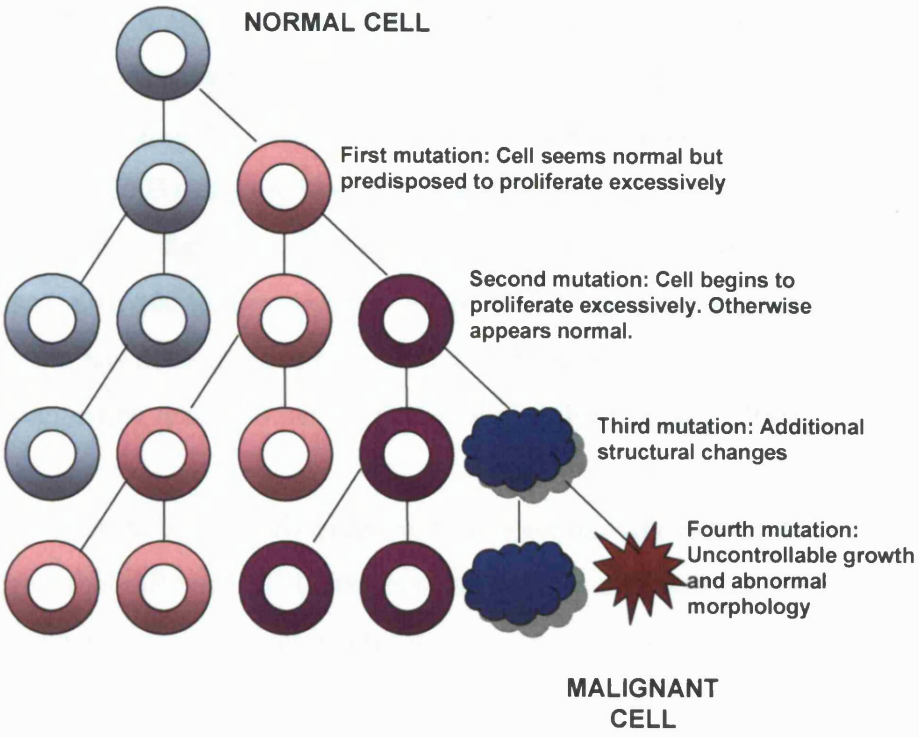


Figure 1.4 Model of malignant transformation in which clonal outgrowth results from the accumulation of multiple genetic aberrations.

The emergence of a cancer cell is thought to arise via a process of clonal evolution in which a daughter cell aquires a cancer-promoting mutation, passes it on to its progeny and all future generations. At some point, one of the descendants acquires a second mutation, and each later descendent acquires sequential ones. Eventually a cell accumulates enough mutations to cross the threshold to cancer.

MLL translocations may follow the model of malignant transformation described with secondary mutations necessary for transformation, or it may be that the MLL-translocation is the only event required to cause leukaemia. Studies have shown that a malignant clone develops in utero which is able to cause *MLL* rearranged leukaemia in concordant twins (Greaves, *et al* 2003), and together with the short latency of infant leukaemia, support that MLL translocation is the only event required to cause disease. In addition, mouse models of MLL leukaemia in which the translocation has been engineered give rise to disease with very short latency suggesting that no other event is required (Forster, *et al* 2003).

However, other mouse models give rise to leukaemia with varying latencies and suggest that different MLL-fusion proteins have different capacities to induce leukaemia and some may have a greater requirement for secondary mutations than others. Such long-latency leukaemias arise in retroviral transduction models of MLL-ELL, MLL-CBP, MLL-AFX and MLL-FKHRL1 (Lavau, *et al* 2000a, Lavau, *et al* 2000b, So and Cleary 2003). MLL-ENL, MLL-AF9, MLL-GAS7, MLL-AF10 and MLL-AF1p cause leukaemias with shorter latencies in mouse models and may be more efficient in their oncogenic action (Cozzio, *et al* 2003, DiMartino, *et al* 2002, Martin, *et al* 2003, So, *et al* 2003a, So, *et al* 2003b).

The MLL fusion may be the primary event that predisposes the cell to further genetic aberrations before leukaemia arises. If secondary mutations are required, these may become independent of the initial primary event of MLL translocation. Conditional MLL-ENL expression in vitro causes immortalization in HPC but these cells differentiate when MLL-ENL expression is turned off (Horton, *et al* 2005, Zeisig, *et al* 2004) suggesting that the immortalized phenotype is dependent on the maintenance of primary MLL-ENL expression *in vitro*. Results of inhibiting MLL fusion expression in cell lines vary. Maintained expression of the fusion protein

appears necessary to sustain the phenotype in blasts harbouring MLL-AF9 which show apoptosis following targeted inhibition of this fusion protein (Kawagoe, *et al* 2001, Pession, *et al* 2003) and similarly targeted inhibition of MLL-ENL in a leukaemic cell line also induced apoptosis (Akao, *et al* 1998). However, THP-1 cells (from a human monocytic cell line) were unable to terminally differentiate following knock down of MLL-AF9 protein in these cells (Pession, *et al* 2003), and this may be due to the harbouring of many further acquired mutations in such cells which may make them independent of MLL-AF9 in regard to their malignant behaviour.

1.4.2 The cell of origin of the malignant clone

Acute leukaemia arises from the clonal expansion of myeloblasts or lymphoblasts in the blood or bone marrow to form AML or ALL respectively (Downing and Shannon 2002). The characteristics of the cell of origin, or 'leukaemic stem cell' (LSC), in which leukaemia-associated translocations arise continue to be investigated. Such a cell might have the characteristics of a HSC with the ability to self-renew, or possibly a progenitor cell with the ability to proliferate and differentiate but without the ability to self-renew (Huntly and Gilliland 2005).

The association of particular lineages of leukaemia with certain MLL fusion proteins gives rise to the possibility that the fusion partner determines the lineage of the leukaemia in a multipotent haematopoietic progenitor. The progenitor cell type may determine its susceptibility to the integration of a particular fusion partner, or the cell type may determne its susceptibility to being transformed by a particular fusion gene (Daser and Rabbitts 2004).

Evidence for the *in utero* origin of a number of fusion-associated paediatric leukaemias has been established (Greaves 2003). Direct evidence for the pre-natal origin of t(4;11) infant ALL was demonstrated by the presence of

unique *MLL-AF4* genomic fusion sequences present in retrospective analysis of neonatal blood spots from individuals who were later diagnosed with ALL (Gale, *et al* 1997) and may suggest the cell type that is targeted in these leukaemias. The mechanisms by which these translocations occur *in utero* is unknown, but it is possible that excess LT-HSC which arise during highly active foetal haematopoiesis may be more prone to apoptosis but are then rescued from cell death (abortive apoptosis). The initiation of DNA cleavage in the initial stages of apoptosis may result in transient reversible double strand breaks and may additionally mediate recombination events resulting in a chromosomal translocation (Khodarev, *et al* 1999). In this way, an occasional cell carrying the fusion gene may survive and subsequently expand to form a pre-leukaemic clone if it has a growth advantage and a permissive environment (Greaves and Wiemels 2003).

Gene expression analyses of MLL-rearranged leukaemias may give clues as to the target cell of origin, and have shown differences in the genes expressed depending on the phenotype of the leukaemia. Genes that are relatively highly expressed in MLL-rearranged ALL are associated with haematopoietic progenitors and developing myeloid cells, while genes expressed at lower levels are associated with lymphoid identity (Armstrong, *et al* 2002). This may suggest that the cell of origin is one with both lymphoid and myeloid potential, such as the MPP. A number of mouse models using primary haematopoietic cells with multilineage potential which have been retrovirally transduced with *Mll* fusion genes have resulted in transformation to a self-renewing biphenotypic or myeloid cell and the development of leukaemia following transplantation (So, *et al* 2003a, Zeisig, *et al* 2004).

It has been proposed that the LSC arises at the level of the normal HSC with its impact being cell-context dependent, and reflecting activation of more primitive cell types (Cozzio, *et al* 2003, Greaves and Wiemels 2003,

Huntly and Gilliland 2005, Reya, *et al* 2001). Normal tissue-specific stem cells and cancer cells share the ability to self-renew, and it has been proposed that newly arising cancer cells result from a deregulation of pathways involved in cell division that are normally expressed by stem cells. Pathways associated with stem cell renewal and cancer development include inhibition of normal apoptosis and over-expression of genes in *Bcl2*, *Notch*, *Sonic hedgehog*, *Wnt* and *Polycomb* signalling pathways, as well as aberrant *Hox* family transcriptional expression (Domen, *et al* 1998, Taipale and Beachy 2001). In view of this association between deregulated self-renewal and malignancy, it is possible that stem cells are the actual targets of transformation in cancer. Such a cell would sustain the growth and spread of tumours while repopulating the distinct types of cell represented within the tumour, and not be subject to the same intrinsic and extrinsic controls as normal stem cells (Huntly and Gilliland 2005). Alternatively, a stochastic model of tumourigenesis proposes that each tumour cell has the potential to self-renew and thus recapitulate the tumour, but each has a low probability of entering the cell cycle and encountering a permissive environment in which to do this (Reya, *et al* 2001). The phenotype of distinct populations within a tumour, and their functional ability to re-capitulate the tumour phenotype will need to be assessed in order to differentiate between these models (Huntly and Gilliland 2005).

Stem cells in the haematopoietic system are in a state of constant, activated self-renewal and persist for long periods of time and are therefore an attractive candidate for the cell of origin in which leukaemic translocations arise. Fewer mutations may be required to maintain self-renewal in the leukaemic cell and there is temporally a greater opportunity for mutations to accumulate in individual haematopoietic stem cells. This is supported by evidence that the *AML1-ETO* fusion transcripts in t(8;21) AML are found in a fraction of normal HSC in the marrow, and that cells generating AML in non-obese diabetic / severe combined immunodeficient (NOD/SCID) mice

have a similar immunophenotype to normal HSC in that they are $CD34^+$ $CD38^-$ (Bonnet and Dick 1997). The LSC in ALL harbouring the breakpoint cluster region-Abelson *(BCR-ABL)* fusion gene has also been demonstrated at the level of the HSC (Cox, *et al* 2004) and distinct patterns of HSC and B-cell progenitor involvement have been shown for the different fusion products of the t(9;22) translocation (Castor, *et al* 2005). TEL-AML1 fusions have been shown to arise in immunophenotypically committed B-cell progenitors rather than in the HSC, and in none of the cases involving BCR-ABL or TEL-AML1 was there expansion of the leukaemic clone at the expense of the normal HSC compartment (Castor, *et al* 2005).

A role for the more restricted progenitor or differentiated cell as the LSC has also been suggested, with activation of oncogenic pathways allowing the progenitor to re-establish properties of self-renewal. This model is supported by the generation of leukaemia from restricted myeloid progenitors overexpressing the B-cell lymphoma 2 (BCL2) protein (Jaiswal, *et al* 2003). A direct role for MLL-ENL in the immortalisation and transformation of a myeloid progenitor has been demonstrated (Lavau, *et al* 1997). Both HSC as well as restricted progenitors transduced with MLL-ENL showed self-renewal properties by forming colonies in serial methylcellulose re-platings and growth in liquid culture, and generated AML in vivo. However, transformation by MLL-ENL of HSC appeared to be more efficient than of CMP as assessed by limiting dilution analysis (Cozzio, *et al* 2003). Similar assays demonstrated expansion of foetal liver myeloid progenitor cells by MLL-AF9 (Johnson, *et al* 2003), and purification and analysis of granulocyte/macrophage progenitors from patients with chronic myeloid leukaemia in blast crisis has also demonstrated self-renewal properties in these cells (Jamieson, *et al* 2004).

Recently it has been proposed that the leukaemic stem cell may arise from overexpression in committed progenitors of genes which normally control

cell cycle regulation in normal haematopoietic stem cells. MLL-AF9 was introduced into committed granulocyte macrophage progenitor cells to confer a self-renewal capacity on these cells through re-activation of only a subset of normal HSC regulatory genes, that resulted in the emergence of leukaemic stem cells that initiated disease (Krivtsov, *et al* 2006).

Evidence supports both a multipotential as well as more restricted cell of origin as the target for MLL translocations, and further studies are required to identify whether the LSC in these cases is characteristic for all resulting leukaemias, or whether it may vary with the fusion gene or leukaemia phenotype.

1.5 MLL-translocations in acute leukaemia

1.5.1 Structure of MLL fusion genes

MLL gene rearrangements include deletions, duplications, inversions and reciprocal translocations at 11q23, and these are implicated in approximately 5-10% of all AML, ALL, biphenotypic leukaemia and myelodysplastic syndrome (MDS) in children and adults. The exon-intron structure of the *MLL* gene reveals a number of recombination-prone sequences within the breakpoint cluster region (BCR) of the gene spanning exons 5-11. Following a reciprocal translocation, the N terminal region of MLL is retained with the AT hooks, nuclear localization signals and transcriptional repression domains, while the C-terminal region with transactivation and SET domains are replaced by the C- terminal region of one of over 40 fusion partners (Ayton and Cleary 2001, Collins and Rabbitts 2002, Eguchi, *et al* 2003). The MLL fusion protein also lacks the Taspase 1 proteolytic cleavage site and the FYRN domain required for interaction with the p180 C-terminal MLL fragment. The most frequently

found fusion genes are *MLL-AF9* [t(9;11)], *MLL-AF4* [(t(4;11)] and *MLL-ENL* [t(11;19)] (Pui, *et al* 2003).

1.5.2 Structure and function of MLL partner proteins

Fusion partner genes associated with MLL leukaemias are shown in Table 2. Most of the fusion partner proteins do not share homology or common functional domains and can be broadly divided into nuclear transcriptional regulators and cytoplasmic signalling molecules (Eguchi, *et al* 2003). However, some common gene families do exist and group together some genes with structural and functional similarity. Such groups include *AF4/AF5q3I/LAF4, ENL/AF9* and *AF10/AF17* (Nakamura, *et al* 1993, Nilson, *et al* 1997, Taki, *et al* 1999). The most common fusion partners found in paediatric leukaemias are AF4, AF9 and ENL and all proteins have regions rich in serine and proline, possess nuclear localisation signals and are able to activate transcription (Djabali, *et al* 1992, Iida, *et al* 1993, Nakamura, *et al* 1993, Prasad, *et al* 1995, Rubnitz, *et al* 1994). AF9 and ENL share homology in both their amino and carboxy terminal regions. These domains are homologous to domains found in the yeast ANC1 protein that associates with the SWI/SNF complex involved in chromatin remodeling through the SNF5 protein (Cairns, *et al* 1996, Welch and Drubin 1994), and ENL has also been identified as a component of a novel human SWI/SNF complex (Nie, *et al* 2003). Similarly the MLL fusion partner AF10 binds to GAS41 which interacts with the SWI/SNF complex via INI1 (Debernardi, *et al* 2002) and AF10 may further be involved in chromatin re-modelling through its interaction with the H3-K79 histone methyltransferase hDOT1L (Okada, *et al* 2005). AF4 and AF9 have been demonstrated to co-localise in novel nuclear foci (Erfurth, *et al* 2004) and the MLL-AF4 fusion protein is also shown to recruit AF9 to these nuclear bodies. ENL has been shown to interact with AF4 among other partners as part of a YEATS supercomplex that interconnects them all to bind to H3 in order to allow productive transcription and is further evidence that these

partners function to influence chromatin remodelling (Zeisig, *et al* 2005). Thus AF4, AF9 and ENL may all be able to directly or indirectly recruit the SWI/SNF chromatin remodelling complex, and the normal interaction of these proteins with each other and with the complex may be disrupted as a result of their involvement in the formation of a fusion protein.

Table 2 Fusion partner genes involved in MLL-associated leukaemias

Gene	Chromosome locus	Functions
Nuclear partner		
AF4	**4q21**	**Transcriptional activator**
AF9	9p22	Transcriptional factor
AF10	**10p12**	**Transcriptional factor**
ELL	19p13.1	RNA polymerase II transcription elongation factor
ENL	**19p13.3**	**Transcriptional activator**
AFX	Xq13	Forkhead transcriptional factor
LAF4	**2q11**	**Transcriptional activator**
AF3p21	3p21	Not known
FKHRL1	**6q21**	**Forkhead transcriptional factor**
CBP	16p13	Transcriptional coactivator, histone acetylase
P300	**22q13**	**Transcriptional coactivator, histone acetylase**
AF17	17q21	Transcriptional factor, upregulated by B-catenin
Cytoplasmic partner		
AF6	6q27	Maintenance of cell–cell junctions and cell polarity
Septin 6	**Xq22**	**Septin family**
ABI1	10p11.2	Regulation of endocytosis, cell motility
LARG	**11q23.3**	**Activation of Rho GTPases**
GAS7	17p13	Actin assembly/crosslinking of actin

		filaments
EEN	**19p13.3**	**Regulation of endocytosis**
FBP17	9q34	Not known
GMPS	**3q25**	**Guanosine monophosphate synthetase**
GRAF	5q31	Negative regulator of RhoA
AF9q34	**9q34**	**Ras GTPase-activating protein**
GPHN	14q24	Gly and GABA receptors assembly
CBL	**11q23.3**	**Negative regulator of receptor tyrosine kinases**
LASP1	17q21	Not known

Apart from nuclear fusion partners, a number of partner genes encode cytoplasmic proteins possessing a number of domains required for protein-protein interactions, but lacking in any transactivation domains. Mechanisms by which such proteins contribute transcriptional activity to the fusion protein are outlined below.

1.5.3 Mechanisms of transcriptional deregulation by *MLL*-fusion genes

Models have been proposed relating to the normal function of *MLL* in the presence of *MLL* fusion genes and are illustrated in Figure 1.5 (Ernst, *et al* 2002). A simple gain-of-function model proposes that normal MLL function is altered to cause overexpression of an MLL target gene involved in cell survival or proliferation. A second model proposes that in addition to gain-of-function of one *MLL* allele, the second allele becomes haploinsufficient with resulting underexpression of a gene normally expressed to negatively regulate survival or proliferation. Finally a dominant-negative model proposes that the *MLL* fusion gene simply intereferes with the normal expression by MLL of a gene which negatively regulates cell survival and proliferation.

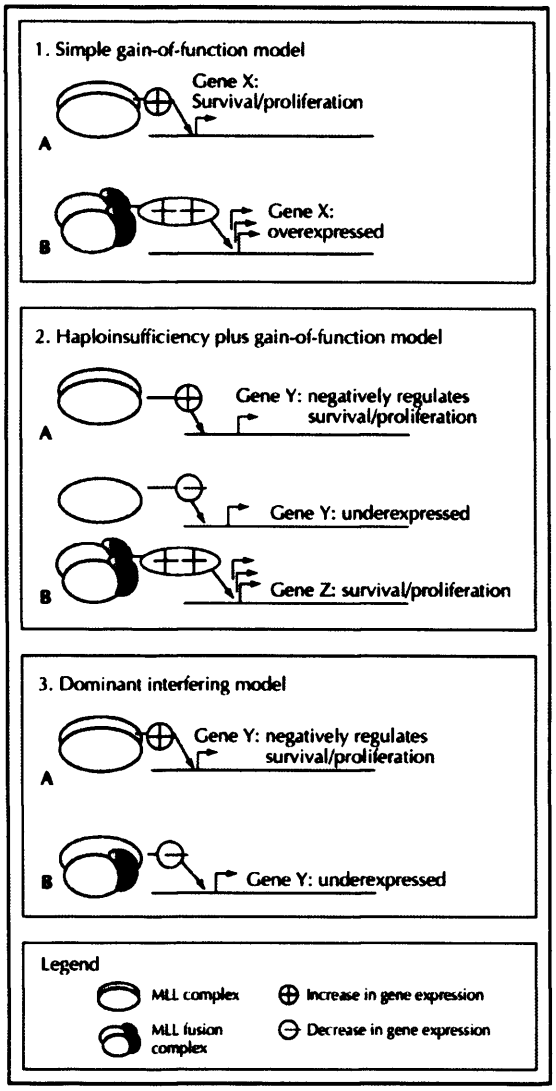


Figure 1.5 Models of how leukaemogenic fusion proteins may confer gain-of-function, gain-of-function and haploinsufficiency or dominant-negative properties on the WT *MLL* allele.

Model 1 shows a simple gain-of-function model where the fusion protein acts to cause overexpression of the cell survival/proliferation gene X. Model 2 shows how haploinsufficiency caused by the fusion protein results in underexpression of gene Y, as well as overexpression of another proliferation/survival gene Z. Model 3 shows how gene Y is underexpressed as a result of inhibition of MLL function by the fusion protein. Reproduced from (Ernst, *et al* 2002).

The transcriptional activity of the MLL fusion protein is likely to be significantly different to that of wild-type MLL as a result of loss of the MLL SET domain, loss of the Taspase proteolytic cleavage site and loss of the PHD domain and the oncogenic potential of the fusion protein may result from this loss-of-function of the normal MLL protein. However, the contribution of the C-terminal domains of the fusion partners may also be responsible for altered transcription of the MLL target genes, or of a different set of target genes, and leukaemia may arise through a gain-of-function of the fusion protein.

The MLL fusion protein may act in a dominant negative manner by inhibiting the normal function of WT MLL protein such that normal targets of MLL are underexpressed. Evidence to support a dominant-negative effect of the MLL fusion protein to inhibit normal MLL function includes the ability of a truncated MLL protein to inhibit the GCSF-mediated differentiation of 32Dcl3 cells, while full length MLL cannot (Joh, *et al* 1996). In addition, fusion of a truncated *Mll* gene to the bacterial LacZ gene was sufficient to cause long-latency leukaemias in chimaeric mice following homologous recombination in ES cells (Dobson, *et al* 2000) suggesting that either truncation or gene fusion itself was able to cause leukaemia *in vivo*. In addition, the ability of the fusion protein to act as a dominant negative inhibitor of the normal function of the partner protein is implied by the suppression of the normal apoptotic function of wild-type forkhead protein FKHRL1 by the fusion of MLL to the forkhead transcription factor AFX (So and Cleary 2002). A similar dominant negative inhibition by the fusion protein may suppress the anti-proliferative functions of the wild-type fusion partners GAS7, ABI1, AF6 and AF1p (So and Cleary 2002).

However, there is also evidence in favour of a gain of function conferred to MLL by the fusion partner. Simple truncation of MLL is not sufficient to immortalize HPC, which neither occurs if the partner protein ENL is simply

overexpressed (Lavau, *et al* 1997). Loss of one *Mll* allele does not result in leukaemia as shown by knock-in mice which possess only one functional copy of *Mll* and express a portion of MLL found in fusion proteins fused to a short MYC tag polypeptide (Dobson, *et al* 1999). If MLL fusions acted by inhibiting endogenous *Mll* function then *Mll-Af9* knock in mice would be expected to show defects similar to those seen in *Mll* $^{-/-}$ or *Mll* $^{+/-}$ mice but these abnormalities are not detected (Corral, *et al* 1996, Dobson, *et al* 1999). Multiple studies using retroviral insertion of *Mll* fusion genes into HPC to generate transformed cells also show the development of leukaemia without disrupting the endogenous *Mll* gene (Lavau, *et al* 1997, So and Cleary 2002). These studies imply that MLL fusions act dominantly over the endogenous MLL protein in a gain of function manner.

In vivo models show that the partner protein appears to confer intrinsic transactivation activity or provides a dimerization potential to the MLL fusion protein resulting in its acquisition of a transformation potential. Both the AT hooks and DNA methyltransferase domains of MLL, as well as the transcriptional activation domain of ENL are required for MLL-ENL mediated transformation (Slany, *et al* 1998). The transactivation region of ENL containing two helical structures is also highly conserved with the AF9 fusion partner. Both the DNA methyltransferase domain of MLL and the transcriptional activation domain of ENL are also required for transactivation of the *Hoxa7* promoter by MLL-ENL (Schreiner, *et al* 1999) and therefore, the domains required for transformation are also those required for transcriptional activation. The methyltransferase domain of MLL which normally represses transcription, is interestingly seen to contribute a transactivation potential when fused to a partner protein. Furthermore, the ability of a transactivation domain in the nuclear MLL fusion partner to confer leukaemic potential is further supported by evidence showing that both MLL-ENL as well as an artificial MLL fusion protein, formed by fusion of the activation domain of VP16 to MLL, causes transactivation of the *Sv40*

promoter and *Hoxa7* (Zeisig, *et al* 2003a). Two other activation domains were less potent than either fusion in transactivating these promoters. In addition, only MLL-ENL and MLL-VP16 were able to immortalize HPC (Zeisig, *et al* 2003a). However, MLL-VP16 transactivated these promoters less efficiently than MLL-ENL and this was reflected in the phenotype of the MLL-VP16 immortalized HPC which did not proliferate as greatly as those of MLL-ENL suggesting a correlation between the degree of transactivation of target genes and the severity of the phenotype (Zeisig, *et al* 2003a). Thus the transactivation domains of the nuclear fusion partner proteins appear instrumental in conferring a leukaemogenic potential to the fusion proteins in a gain of function manner.

Cytoplasmic fusion partners such as AF1p, GAS7 and SEPTIN6 do not have any intrinsic transactivation capacity but possess coiled coil domains which are able to facilitate oligomerization (Ono, *et al* 2005, So, *et al* 2003b). The fusion of LacZ protein to MLL also confers a transforming capacity which may be due to the oligomerization of LacZ protein, rather than leukaemia occurring as a result of MLL truncation alone as was originally thought (Dobson, *et al* 2000). The latency of these leukaemias was very long, compared to that in mice expressing the MLL-AF9 transgene, suggesting that the mechanisms of leukaemogenesis in each case may be different. The normal B-galactosidase protein exists in tetrameric form and it was thought that MLL-LacZ fusion may therefore also exist as a tetramer (Dobson, *et al* 2000).

When fused to MLL both AF1p and GAS7 confer transcriptional activation activity to the MLL fusion protein such that it was able to transactivate the *Hoxa7* promoter (So, *et al* 2003b). Again it appears that truncated MLL with its transrepression domain is converted into a transactivator following fusion to a partner protein. Interestingly, both the cytoplasmic partner fusion MLL-GAS7 and the nuclear partner fusion MLL-ENL transactivated a similar

subset of *Hox* genes to cause immortalization of HPC (So, *et al* 2003b). Such target genes may represent a critical subset required for leukaemogenesis in the context of an MLL fusion protein.

A possible mechanism by which MLL target genes are deregulated may involve Menin, the tumour suppressor protein product of the *MEN1* gene. This is essential for embryonic development, and mutation leads to multiple endocrine neoplasms (Biondi, *et al* 2004). The mechanisms by which Menin acts as a tumour suppressor are unclear, but it is a co-factor to MLL within a histone methyltransferase complex including ASH2, RBBP5, and WDR5, and appears to directly regulate expression of the cyclin-dependent kinase inhibitors p27Kip1 and p18Ink4c (Milne, *et al* 2005a). Furthermore, Menin's function as a tumour suppressor gene may be related to its regulation by histone methyltransferase activity of Hoxc6 and Hoxc8 as differentiation-regulating genes (Hughes, *et al* 2004), and it appears critical for the maintenance of *Hox* gene expression by MLL in normal development (Yokoyama, *et al* 2004). The role of Menin as a co-factor for MLL in regulating normal *Hox* gene expression may allow insight into the mechanism by which the MLL fusion proteins cause leukaemia by abberantly expressing Hox genes. This is supported by the finding that MLL fusion proteins retain the ability to stably associate with Menin, and cause aberrant expression of *Hox* genes in MLL fusion transformed leukaemic blasts, which is then reversed on targeted deletion of Menin, with reversal of the differentiation arrest (Yokoyama, *et al* 2005). Menin, MLL and RBBP5 localise to the promoters of thousands of human genes but do not always bind together, suggesting that Menin is a general regulator of transcription (Scacheri, *et al* 2006).

1.6 Potential targets of MLL-fusion genes in acute leukaemia

1.6.1 The role of *Hox* genes in MLL-fusion leukaemia

The possibility that *Hox* genes may be a target of MLL fusion proteins is suggested by their role in the transcriptional control of normal haematopoiesis under MLL regulation as described above. The implication of *HOX* genes in causing human leukaemia is suggested by the overexpression of certain *HOX* genes in both AML and ALL, and by the finding of HOX proteins as fusion partners of leukaemogeneic proteins.

HOXA and *HOXB* genes co-express in AML associated with standard or high risk (Drabkin, *et al* 2002), and *HOXA9* expression was identified through micoarray analysis of ALL and AML as associated with poor prognosis in human AML (Golub, *et al* 1999).

HOXA9 has also been identified as a fusion partner of the nucleoporin gene *NUP98* in the AML patients with t(7;11) translocation, and retroviral gene transfer of this fusion results in a myeloproliferative disorder (Kroon, *et al* 2001). Acceleration of myeloproliferative syndrome (MPS) to AML was observed following co-expression of the fusion with *Meis1* further supporting the *Hoxa9* and *Meis1* interaction in leukaemogenesis (Kroon, *et al* 2001). A number of other *HOX* genes have been identified as fusion partners of *NUP98* in patient AML including *HOXA11, HOXA13, HOXC11, HOXC13* and *HOXD13* (Pineault, *et al* 2004). HOX partner proteins HOXA10 and HOXB3 are not found in human disease but are still leukaemogenic in mouse models when fused to NUP98 (Pineault, *et al* 2004) suggesting a common mechanism for oncogenic potential shared by most *Hox* genes when aberrantly expressed. However, *Hoxb4* has previously been shown to fail in its ability to cause leukaemia in retroviral transduction/transplantation models (Sauvageau, *et al* 1995, Thorsteinsdottir, *et al* 1999) and neither is

the NUP98-HOXB4 able to generate leukaemia in comparison to other NUP98-HOX fusion proteins. There therefore appears to be a definite leukaemogenic potential in certain, but not all, *Hox* genes, and the differences between the two groups may be critical to an understanding of leukaemia mediated by aberrant *Hox* gene expression.

The *Hox* gene transcriptional co-factor *Meis1* does not contain a homeodomain but appears to co-operate with *Hoxa7, Hoxa9, Hoxa10, Hoxb3, Hoxb4* or *Hoxb6* to induce leukaemia in mouse models (Kroon, *et al* 1998, Thorsteinsdottir, *et al* 2001, Thorsteinsdottir, *et al* 1997). Overexpression of *Hox*a9 and *Meis1* in HPCs results in immortalization of a myeloid lineage HPC (Calvo, *et al* 2000) and Meis1 may increase the proliferative or self-renewal capacity of cells which have undergone a differentiation block as a result of *Hoxa9* expression (Pineault, *et al* 2005). This may explain the dramatically shorter latency of leukaemias which develop when *Meis1* is also overexpressed with *Hox*a9 in HPC in comparison to *Hoxa9* expression alone, though the phenotype of the resulting leukaemia is unchanged by *Meis1* overexpression (Thorsteinsdottir, *et al* 2001). *Hoxa9* and *Meis1* co-expression also protected myeloid and lymphoid cell lines from apoptosis (Wermuth and Buchberg 2005) and this may also be another mechanism by which these genes co-operate to promote leukaemogenesis.

Studies of gene expression profiles of patient samples harbouring *MLL* rearrangements have identified *HOX* genes as fundamental to a unique signature that differentiates these leukaemias from those lacking MLL translocations (Armstrong, *et al* 2002). Lymphoblastic leukaemia with an *MLL* rearrangement exhibits increased expression of a number of *HOXA* genes *HOXA4*, *HOXA5*, *HOXA9*, *HOXA10*, *HOXC8* and the *HOX* interacting gene *MEIS1*, but not *HOXA7* when compared to other ALL (Armstrong, *et al* 2002, Tsutsumi, *et al* 2003, Yeoh, *et al* 2002). In MLL-rearranged AML patient samples, *HOX* genes were also overexpressed compared to non MLL-

rearranged disease and *HOXA4, HOXA5, HOXA9, HOXA10, MEIS1* and *PBX3* were particularly highly expressed (Debernardi, *et al* 2003, Ross, *et al* 2003). However, co-expression of *HOXA9* and *MEIS1* is seen in a number of myeloid leukaemia cell lines regardless of *MLL* status (Lawrence, *et al* 1999) By including three T cell lineage *MLL*-rearranged ALL in a gene expression profiling study some key differences between B cell and T cell lineage *MLL*-rearranged ALL were identified in terms of overexpression of *HOXA9, HOXA10, HOXC6, MEIS1* and *PBX3* (Ferrando, *et al* 2003).

In vitro studies using cell lines which inducibly express the fusion protein are more likely to examine targets involved in the initiation of leukaemia as these cells will not harbour multiple genetic aberrations that may have been acquired. Inducible expression of MLL-AF9 in 32Dcl3 cells demonstrated over-expression of *Hoxa7*, *Hoxb7* and *Hoxc9* genes, although characterisation of the phenotype of the cells was not described in this study (Joh, *et al* 1999). Expression of MLL-AF9 in a leukaemic cell line was associated with maintained levels of *Hoxa7* and *Hoxa10*, and down regulation of these genes followed down-regulation of the fusion protein with resultant apoptosis (Kawagoe, *et al* 2001). Inducible expression of MLL-ENL in an oestrogen-regulated conditional model showed down-regulation of *Flt3, Meis1, Hoxa9* and *Lmo2* when MLL-ENL expression was turned off in the absence of tamoxifen (Zeisig, *et al* 2004). These cells, which were immortalized in the presence of MLL-ENL showed subsequent differentiation. In order to differentiate the gene expression profile resulting directly from loss of MLL-ENL expression from that resulting from cell differentiation alone, a Tetracycline-regulatable model of MLL-ENL expression showed that multiple *Hoxa* genes were down-regulated when MLL-ENL was turned off in the presence of doxycycline but maintained in differentiating cells which continued to express MLL-ENL (Horton, *et al* 2005). These results show that MLL-ENL directly maintains expression of a

subset of *Hox* genes to sustain the immortalized phenotype and contribute to leukaemogenesis.

The role of *Hox* genes and their co-factors in MLL-fusion dependent immortalisation of bone marrow progenitor cells using knock out, retroviral transduction/ transplantation and inter-chromosomal recombination models suggests that overexpression of *Hoxb8, Hoxa9, Hoxa10* or *Hoxb3,* but not *Hoxb4,* will eventually result in AML after several months of latency. *Hoxa9* was of interest as the first *Hox* gene to be disrupted by a recurrent chromosomal translocation in some human leukaemias (Nakamura, *et al* 1996b) and it was initially described as essential for induction of MLL fusion-associated myeloid leukaemia *in vivo* in a bone marrow transduction/transplantation model (Ayton and Cleary 2003). In this study, *Hoxa9* and *Hoxa7* were thought to be specifically required for efficient immortalisation of primary bone marrow cells by MLL-ENL. In the absence of MLL-ENL, expression of these *Hox* genes was lost, and in a conditional MLL-ENL expression system *Hoxa9* and *Hoxa7* deficient progenitors failed to immortalise efficiently (Ayton and Cleary 2003). *Meis1* was initially identified as a gene adjacent to a common retroviral integration site found in murine myeloid leukaemias, and is frequently co-overexpressed with *Hoxa7* and *Hoxa9* in these leukaemias (Nakamura, *et al* 1996a) and combined action of these proteins may more effectively regulate a critical set of target genes. However, further studies in which leukaemia occurred following transplantation of *Hoxa9* deficient progenitors into an *Mll-Af9* knock-in mouse suggested that the phenotype and malignant potential of the transformed cell is rather defined by the expression pattern of several *Hox* genes (Kumar, *et al* 2004). Furthermore, leukaemic transformation occurred in the absence of either *Hoxa9* or *Hoxa7* in a retroviral transduction model (So, *et al* 2004).

In summary, MLL fusions do not appear to regulate any particular *Hox* gene but rather a *Hox* combinatorial code appears important in the immortalisation of cells by MLL fusions. In addition, the *Hox* genes are known to require MLL for their maintenance rather than initiation of expression, suggesting that alteration of their expression levels by MLL fusions may not be the initiating event in MLL fusion-mediated leukaemogenesis.

1.6.2 The role of other candidate targets of MLL-fusion proteins

Microarray studies have highlighted wild-type FLT3 overexpression as a distinguishing feature of *MLL*-rearranged ALL compared to *MLL*-non-rearranged ALL and AML (Armstrong, *et al* 2002). FLT3 is a receptor tyrosine kinase expressed by immature haematopoietic cells and, together with its ligand, is important for the normal development of stem cells and the immune system. Mutations of *FLT3* have been detected in about 30% of adult patients with AML and a small number of patients with ALL or MDS, and tend to confer a poor prognosis. Activating mutations are found in approximately 25% of paediatric AML patients, but interestingly, *FLT3* point mutations have been found to be particularly common in infant and childhood *MLL*-rearranged leukaemas, with 5 point mutations being found in 30 patients (17%) (Armstrong, *et al* 2003). Such mutations were found in 8 (18.2%) of 44 infants with ALL with *MLL*-rearranged leukaemia (Taketani, *et al* 2004). The possible cooperation of FLT3 mutations and MLL fusion proteins was suggested by induction of an acute myeloid leukemia with short latency when HPC were transduced with both genes only, with subsequent upregulation of *Hoxa7* and *Hoxa9* (Ono, *et al* 2005).

A number of ALL-cell lines harbouring gene rearrangements have been shown to express MEIS1, HOXA9 and FLT3 (Quentmeier, *et al* 2004). Microarray analysis of AML cells with and without *MLL* mutations has

highlighted overexpression of *BEX1* (brain expressed X-linked 1) in MLL-rearranged cell lines (Quentmeier, *et al* 2005).

1.7 Down-regulation of gene expression by shRNA

In order to validate candidate target genes of MLL fusion proteins, the effect of down-regulating their expression by RNA interference can be examined.The process of RNA interference (RNAi) uses double stranded RNA to induce sequence-specific post-transcriptional gene silencing as part of an evolutionarily conserved mechanism present throughout a range of organisms. This process relates to a normal defence against viruses and the mobilisation of transposable genetic elements (Leung and Whittaker 2005). As part of this defence, the natural process in mammals is associated with the induction of interferon (IFN) leading to non-specific translational shutdown and apoptosis (Gil and Esteban 2000). In mammalian cells the biological pathway has been harnessed to knock down the expression of specific genes either by introducing synthetic double stranded small interfering RNAs (siRNAs) of 21-23 base pairs, or by the delivery of plasmid or viral vectors that express double stranded short hairpin RNAs (shRNAs) that are subsequently processed into siRNAs. The shRNAs connect sense and anti-sense strands by a loop and are expressed as single molecules, and rapidly form a hairpin structure with a stem and loop before being cleaved to produce siRNAs. The non-specific cellular activity of the stress response is circumvented by these smaller molecules entering the RNAi pathway further downstream. The effectiveness of siRNA silencing is sequence-specific and thus rules for siRNA design have been developed. The short double stranded RNAs are processed in the cytoplasm by an RNase III-like protein known as a Dicer which cleaves them into small RNA duplexes of 19-25 base pairs, with characteristic 3'-dinucleotide overhangs. These are then incorporated into a multiprotein complex known as the RNA induced silencing complex (RISC), whereupon an ATP-dependent helicase unwinds the duplex,

allowing one of the two strands to independently recognise the mRNA. Site-specific cleavage of the message in the region of the siRNA-mRNA duplex then follows. A schematic overview of the mechanism of shRNA-mediated silencing of gene expression is shown in Figure 1.6A.

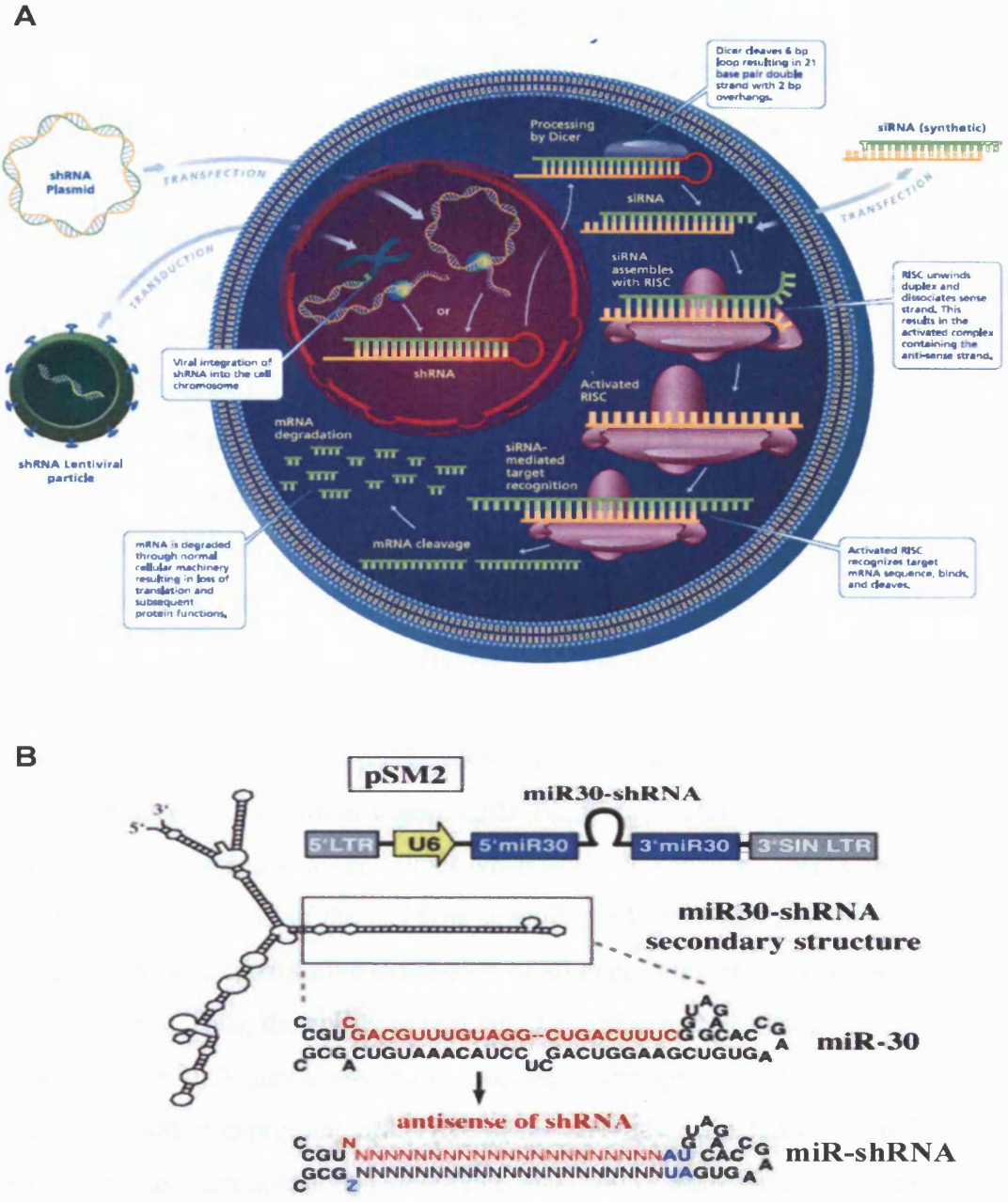


Figure 1.6 Overview of mechanism of inhibition of gene expression by shRNA.

A shows a schematic diagram of the mechanism of shRNA interference with gene expression.

B shows the structure of microRNA (miR-30) in the pSM2 retroviral vector. The miR-30 was modified to express a shRNA of interest (miR-shRNA). Reproduced from (Stegmeier, *et al* 2005).

Amplification of the siRNAs occurs by the RNA-dependent RNA polymerases which are lacking in mammalian cells, and their co-transfection with siRNA duplexes results in transient gene silencing whose persistence depends on the proliferative status of the cells (Leung and Whittaker 2005). Thus, vector-based systems containing RNA polymerase III promoters have been developed and appear to allow the introduction, and the more stable and effective expression, of siRNA in target cells, as well as more potent induction of RNAi than siRNA, and these can also express shRNAs. The human U6 snRNA promoter is the best studied polymerase III promoter and is frequently used in RNAi expression vectors. However, a system of shRNA expression from polymerase (pol) II, (rather than polymerase III), promoters when embedded in a microRNA (miRNA) context has been shown to display very efficient knock-down of gene expression (Stegmeier, *et al* 2005).

The structure of miRNA in the pSM2 vector, and its modification to express a shRNA of interest is shown in Figure 1.6B. Pol III promoters are constitutively expressed in all cell types whereas tissue-specific expression of the shRNA under control of the cell type specific pol II promoter may be more desirable, and constitutive expression of all genes may be toxic to the transduced cells. Thus, the ability to regulate the timing and levels of expression of shRNA may allow more effective downregulation of target genes. A method of expressing microRNA (miR) 30-based shRNAs from pol II promoters has been described (Stegmeier, *et al* 2005). Retroviral, lentiviral or adenoviral vector systems allow greater ease and efficacy of infection, and more stable expression of shRNA constructs than plasmids. Lentiviral vectors are derived from human immunodeficiency virus (HIV)-1 and can infect both dividing and non-dividing post-mitotic cells and successful lentiviral delivery of shRNAs to eukaryotic cells in vitro has been reported by several groups (An, *et al* 2003, Tiscornia, *et al* 2003).
In relation to the use of haematopoietic cells, delivery by lentiviral vectors incorporating the spleen focus forming virus (SFFV) long terminal repeat

(LTR) sequences, together with the Woodchuck hepatitis virus posttranscriptional regulatory element (WRE) has been shown to produce highly efficient long-term expression of a transgene (Demaison, *et al* 2002). Such a vector was used in this study to drive the expression of microRNA expression of shRNA against a gene of interest and a green fluorescent protein (GFP) reporter.

1.8 Aims

The aims of the project are outlined below:

- to identify candidate target genes of MLL-ENL and MLL-AF9 in the 32Dcl3 mouse myeloid progenitor cell line, in order to identify genes which may be useful targets for biological therapy following further analysis

- to determine the phenotypic and functional characteristics of 32Dcl3 cells expressing these MLL fusion proteins

- to determine whether the mechanism by which MLL fusion proteins function confer the leukaemic phenotype on haematopoietic progenitor cells is according to a gain-of-function or dominant-negative model

2 Materials and Methods

Chemicals were purchased from VWR, Poole, Dorset, UK unless otherwise stated. Tissue culture reagents were purchased from Invitrogen, Paisley, UK unless otherwise stated. Restriction enzymes were purchased from Promega, Madison, Wisconsin, USA. Primer/probe sets for Q-PCR were purchased from Applied Biosystems, Foster City, USA. All recombinant cytokines were purchased from Peprotech EC, London, UK.

Table 3 Buffers and Solutions

Buffer	Components
Erythrocyte lysis buffer	17 mM Tris (pH 7.2); 0.144 M NH_4Cl
Stain buffer (flow cytometry)	PBS; 0.5% BSA; 0.05% sodium azide
RIPA lysis buffer	150 mM NaCl; 1% Triton X-100; 0.5% sodium deoxycholate; 0.1% SDS; 50 mM Tris (pH 8.0); 5 mM EDTA
NP40 lysis buffer	150 mM NaCl; 0.5% NP40; 50 mM Tris (pH 8.0)
5 x protein sample buffer	500 mM DTT; 10% SDS; 312.5 mM Tris (pH 6.8); 0.05% Bromophenol Blue; 25% Glycerol
2 x protein sample buffer	200 mM Dithiothreitol (DTT), 80% SDS, 10% glycerol[Sigma], 4% bromophenol blue, 200 mM Tris pH6.8
1 x running buffer	0.192 M glycine; 25 mM Tris; 0.1% SDS
1 x transfer buffer	9.5 mM CAPS (pH 11.0)
1 x stripping buffer	62.5 mM Tris (pH 6.8); 1% SDS; 50 mM 2-mercaptoethanol

TNES	**TE (pH 8.0); 0.1 mM NaCl; 1% SDS**
Denaturing solution	1.5 M NaCl; 0.5 M NaOH
Neutralising solution	**1 M Tris (pH 7.4); 1.5 M NaCl**
5 x probe labelling buffer	0.5 M Na_2HPO_4; 0.5 M NaH_2PO_4; 0.5% 2-ME; 1 mM of dATP, dGTP and dTTP
Pre-hybridisation solution	**5 x SSC; 0.5% SDS; 0.1 g/ml dextran sulphate; 5 x Denhardt's solution**
TE (pH 8.0)	10 mM Tris (pH 8.0); 1 mM EDTA (pH 8.0)
Luria-Bertani (LB) broth	**1% tryptone, 0.5% yeast extract, 1.0% NaCl (pH 7.0)**
LB agar	LB broth medium plus 15g/l agar
TAE buffer	**40 mM Tris-acetate, 1 mM EDTA at pH 8.3**
Electrophoresis buffer	25 mM tris-Hcl (pH 8.3), 192 mM glycine, 0.1% SDS
TBS	**20 mM Tris-HCl (pH 7.5), 150 mM NaCl**
TBS-T	20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1 % Tween 20 [Sigma]
10 x SSC	**3M NaCl, 0.3 M sodium citrate (pH 7.0) [National Diagnostics]**
HotSHOT Alkaline Lysis reagent	25 mM NaOH, 0.2mM EDTA
HotSHOT Neutralisation buffer	**40 mM Tris-Hcl (pH 5.0)**

2.1 Culture of 32Dcl3 cell lines

Cells were maintained at 37°C with 5% CO_2 in a humidified atmosphere.

32Dcl3 cells

32Dcl3 cells were obtained from the American Type Culture Collection (ATCC) and maintained in liquid culture in Iscove's Modified Dulbecco's Media (Sigma, Poole, UK) containing 10% fetal calf serum (FCS) (Globepharm, Surrey, UK), 10% conditioned interleukin-3 (IL-3) media derived from cultured WEHI-3B cells, 1% penicillin/streptomycin (100 units/ml penicillin, 100ug/ml streptomycin), and 1% (2mM) L-glutamine.

32DTeton cells

Tetracycline-inducible 32Dcl3 clones were maintained in the above media and were additionally selected and maintained in media containing genetecin at 2.5mg/ml.

32DMAF9 and 32DMENL cells

Tetracycline-inducible 32Dcl3 clones containing the *MLL* fusion genes were additionally selected and maintained in media containing genetecin 2.5mg/ml.and hygromycin 1.2mg/ml.

WEHI-3B cells

WEHI-3B is a leukaemic cell line containing activating retroviral integrations adjacent to the *Hoxb8* and *Il-3* genes. These cells were maintained in DMEM medium containing 10% FCS, 100units/ml penicillin, 100ug/ml streptomycin and 2mM L-glutamine. WEHI-3B cells grew as a mixture of suspension and adherent cells and conditioned medium from these cells was used as a source of IL-3. To split the cells, the suspension cells were harvested, spun down and resuspended in fresh medium. To obtain conditioned medium, the cells were grown for 3 to 4 days until confluent, the

suspension cells removed by centrifugation and the conditioned medium filter sterilised using a 0.22um filter (Millipore, Watford, UK) and stored at -20°C in 50ml aliquots.

2.2 Plasmid constructs

All of the MLL fusion plasmid constructs used in the study were made and sequenced by Dr. Dale Moulding and Dr. Inusha de Silva of the Molecular Haematology and Cancer Biology (MHCB) unit. A diagram of the plasmid constructs is shown in Figure 2.1. The pTRE-MLL-ENL, pTRE-MLL-AF9 and pTRE-MLL-AF4 constructs were all deleted for amino acids 1-26 of MLL. The pTet-tTS-Hyg^r construct was made by digestion of pTRE- Hyg^r with *Xho*1 to release the neomycin resistance cassette, and ligation of this into the *Xho*1 site of pTet-tTS.

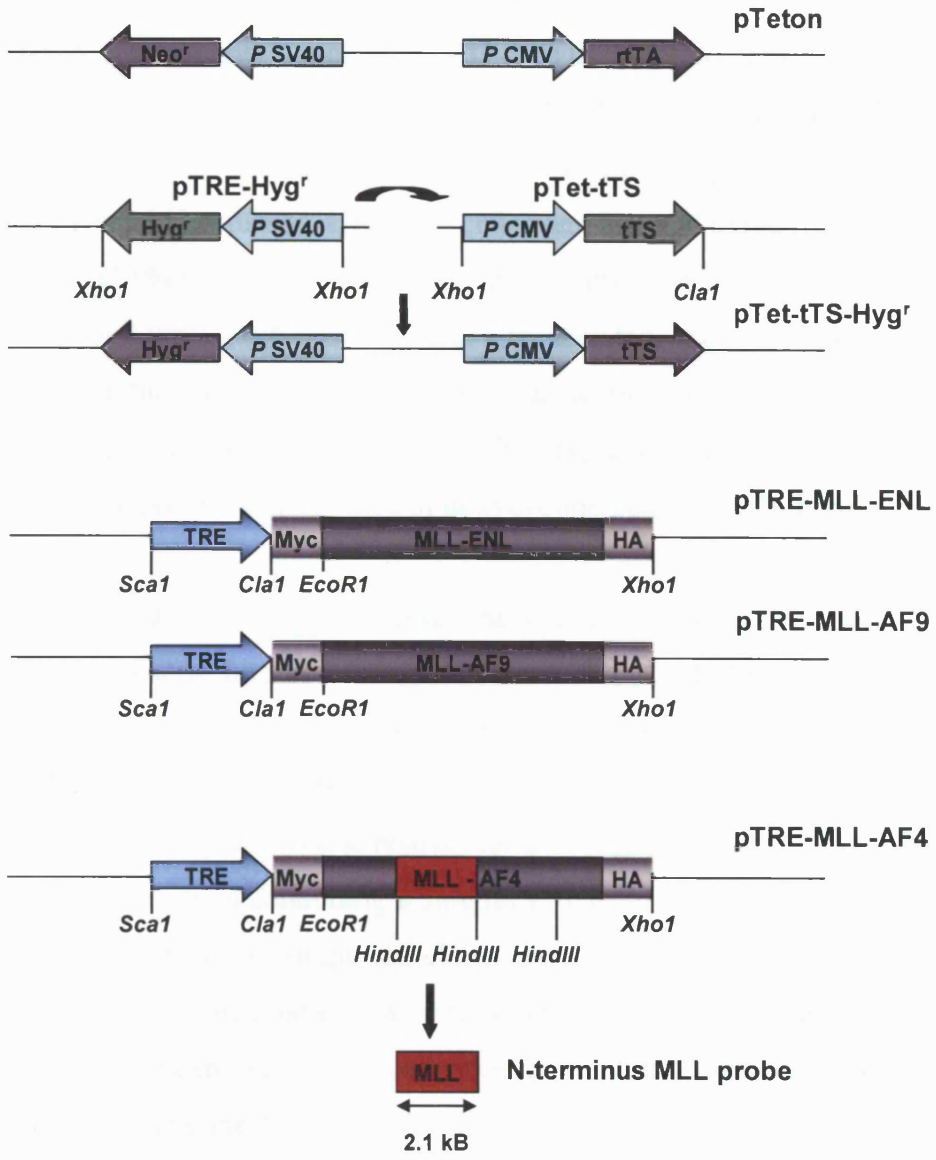


Figure 2.1 A diagram of plasmid constructs used in this study.

*P*SV40: simian virus 40 promoter, Hyg^r: hygromycin resistance gene, *P*CMV; CMV promoter , Neo^r: neomycin resistance gene, TRE: tetracycline response element, tTS: tetracycline suppressor. MYC: myeloblastosis oncogene protein c-MYC tag, HA: haemagluttinin tag.

2.3 Electroporation of cells with plasmid DNA

To generate tetracycline-inducible 32Dcl3 cells (32DTeton) cells, 5 x 10^6 32Dcl3 cells were electroporated in 250ul of IMDM containing 20ug of pTet-On at 260V/950uF using the Gene Pulser ® II electroporation system (BioRad, Hemel Hempstead, UK). The cells were allowed to recover for 24 hours at 37°C/5% CO2 prior to selection in medium containing 2.5mg/ml genetecin for 2 weeks. Single cell clones were isolated, expanded and maintained in the same medium. Transient transfection of a luciferase reporter gene was used to screen for inducible expression after cells were cultured in the presence or absence of doxycycline 2ug/ml for 24 hours.

To generate 32DTeton which expressed the *MLL* fusion genes, 15 x 10^6 32DTeton cells were further electroporated in 750ul of IMDM containing 60ug of pTRE-MLL-AF9 and ptTS-HygR in varying ratios, or 60ug of pTRE-MLL-ENL and ptTS-HygR in varying ratios, or 60ug ptTSHygR alone. The cells were allowed to recover for 24 hours at 37°C/5% CO2 prior to selection in medium containing 2.5mg/ml genetecin and 1.2mg/ml hygromycin for 2 weeks. Single cell clones were isolated, expanded and maintained in the same medium. Western blotting was used to screen for inducible protein expression after cells were cultured in the presence or absence of doxycycline 2ug/ml for 24 hours.

2.4 Isolation of single cell clones

Single cell clones were generated from tetracycline-inducible mixed populations of cells after FACS sorting. Mixed populations of inducible cells were centrifuged and resuspended at 0.5 x 10^5/ml in IMDM medium containing 10% FCS and 10% WEHI-3B conditioned medium. The Autoclone® sorting option on the EPICS® ALTRA™ FACS sorter (Beckham Coulter, High Wycombe, UK) was used to dispense a single live

cell into each well of a 96-well round-bottomed plate containing 100ul of complete medium with appropriate antibiotics for selection. Cells were subsequently propagated and expanded.

2.5 Luciferase reporter constructs and assays

Luciferase assays were performed using the Dual Luciferase Assay system (Promega) according to the manufacturer's recommended procedures. 5 x 10^6 cells 32DTeton clones were electroporated in 250ul IMDM containing 18ug of the firefly luciferase reporter construct and 2ug of pRL-CMV at 260V/950uF. Cells were allowed to recover for 24 hours prior to culturing with or without 2ug/ml doxycycline for 24 hours. Luciferase activity was then measured using the Lumat LB 9507, Perthold Technologies, Austria.

2.6 Preparation of cellular extracts for SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) for protein resolution was performed according to standard protocols.

1 x 10^6 cells were harvested and washed twice in cold PBS. Cells were lysed in either 2 x Lysis Buffer at 60ul per 1 x 10^6 cells, or NP40 lysis buffer containing protease inhibitors (Roche). Cell lysates containing 0.25 x 10^6 cells were resolved by electrophoresis in 7% SDS-PAGE gels using Mini Protean III apparatus (BioRad) according to manufacturer's instructions. Gels were electrophoresed in SDS-PAGE electrophoresis buffer at 150V.

2.7 Protein transfer to PVDF

Protein transfer to polyvinylidene fluoride membrane (PVDF)
Proteins in SDS-PAGE gels were transferred on to PVDF membrane (Millipore) in protein transfer buffer using a BioRad Mini Trans-Blot

Electrophoretic Transfer system according to the manufacturer's instructions. Gels were transferred for 1 hour at 100V at 4°C.

2.8 Western analysis for protein expression

All antibodies were diluted in 5% milk powder in TBS-T. Working dilutions of antibodies used for Western blotting analysis are given in Table 4. Membranes were incubated for 16 hours at 4°C with gentle agitation in 10% milk powder in TBS-T. Membranes were subsequently incubated with primary antibody for 2 hours at room temperature with gentle agitation. Membranes were washed in TBS-T for 1x 20 minute wash and 2 x 10 minute washes. Membranes were then incubated for 1 hour with the relevant secondary antibody. Membranes were then washed in TBS-T for 3 x 15 minute washes. Proteins were visualised by ECL Plus™ reagent (Amersham Biosciences, Little Chafont, U.K). Equal protein loading was checked either by staining the membranes with Ponceau S (Sigma-Aldrich) or examining the expression by Western blot of an appropriate control protein.

Table 4 Antibodies used for Western blotting

Antibody	Clone	Supplier	Working dilution
Mouse anti-Myc	9E10	Roche	1:400
Mouse anti-Myc	9B11	Cell signalling technologies	1:1000
Anti-HSP 90 polyclonal	N/A	Cell Signalling technologies	1:2000
Rat anti-HA	3F10	Roche	1:500
Mouse anti-MLL	N4.4	Dr. M.L Cleary, Stanford.	1:3
Rat anti-tubulin	YL1/2	Serotec	1:1000
Sheep anti-mouse HRP	N/A	Amersham Biosciences	1:2000
Goat anti-rabbit HRP	N/A	Amersham Biosciences	1:2000
Sheep anti-rat HRP	N/A	Serotec	1:2000

2.9 Isolation of genomic DNA from cells

10 x 10^6 cells were washed in PBS and lysed overnight in TNES (400 ul per 10 x 10^6 cells) containing 0.5mg/ml proteinase K (Roche) at 37°C. Genomic DNA was extracted using Phenol-Chloroform-Isoamylalcohol (25:24:1) (Sigma-Aldrich). 0.5ml Phenol-Chloroform-Isoamylalcohol (25:24:1) was added to the 400ul lysate and shaken vigorously for 1 minute. The phases were separated by centrifugation at 16 000 x g for 5 minutes and the supernatant transferred to a separate tube containing 2 x volume of ethanol: 3M NaOAc (25:1). Large precipitates of genomic DNA were lifted from solution using an inoculation loop and carefully washed in 70% ethanol. The DNA was re-dissolved in TE (pH 8.0).

2.10 Southern blotting analysis

Southern blot analysis of DNA was performed according to standard procedures. A hybridisation robe was prepared by digesting pTRE-MLL-AF4 with *Hind* III. This released a 2.1 kb cDNA fragment which contained the N-terminal MLL sequence. This fragment was isolated and gel purified for use in probing the membrane. The probe (100ng) was denatured at 94 °C for 10 minutes and then labelled with [32P] α-dCTP using random primer labelling. The components of the labelling reaction were 5 x labelling buffer (0.25 M sodium phosphate buffer, 0.5% 2-ME and 1 mM of dATP, dGTP and dTTP), 100ng hexanucleotide primers (Sigma-Aldrich), 0.4 mg/ml BSA, 20 mM MgCl2, 5 units Klenow (Promega), 100ng denatured probe and 1.11 MBq 32P dCTP. The labelling reaction was carried out at 37 °C for 2 hours and then stopped by the addition of 0.1% SDS in TE. The probe was purified using Sephadex G50 columns equilibrated with 0.1 M EDTA. The probe was eluted from the column by centrifugation at 390 x g for 3 minutes into 1 M NaOH to denature the probe. The probe was then neutralised by the addition of 2 M Tris (pH 8.5).

Prior to hybridisation, the membrane was blocked by incubation with pre-hybridisation solution containing 0.5mg/ml salmon sperm DNA (Sigma-Aldrich) for 2 hours at 65 °C. The probe was then added and allowed to hybridise to the membrane overnight at 65°C. The membrane was then washed twice in 2 x SSC, 0.1% SDS at 55 °C and bands were visualised using a Typhoon phosphorimager (Amersham Biosciences).

2.11 IL-3 withdrawal experiments

32DMENL cells were seeded to a density of 4 x 10^5/ml in fresh medium at 12 hours prior to the IL-3 withdrawal experiments. Cells were pelleted and washed twice in IL-3 depleted medium (IMDM containing 10% FCS, 2mM L-glutamine, 100 U/ml penicillin and 100 ug/ml streptomycin), and resuspended in IL-3 depleted medium at a density of 4 x 10^5/ml. prior to staining with propidium iodide, 0.5 x 10^6 cells were fixed in ethanol at appropriate time points. The cells were washed in PBS and resuspended in 200ul PBS then added drop-wise to tubes containing 2ml of 70% ethanol at -20°C and left on ice for 1 hour. Cells were then centrifuged at 200 x g for 5 minutes at 4°C, resuspended in 800ul PBS and incubated with 50ul RnaseA (1mg/ml stock) and incubated at room temperature for 20 minutes. Finally cells were stained with 50ul of propidium iodide (50ug/ml stock) and left 10 minutes before flowcytometric analysis using EPICS ® XL ™ flow cytometer and EXPO ™32 software (Beckman Coulter).

2.12 Cytospin analysis of cellular morphology

Approximately 5 x 10^4 cells were washed in PBS and resuspended in 100ul PBS. Cells were centrifuged onto slides at 500rpm for 5 minutes at low deceleration using a cytospin 3 machine (Shandon). The slides were then fixed and stained with May-Grunwald-Giemsa (MGG) using a Shandon

variation 24-4 automated staining machine in the Haematology Department at Great Ormond Street Hospital.

2.13 G-CSF induction of cellular differentiation

Cells were plated out at a density of $2x10^5$/ml in fresh medium. After 12 hours cells were re-adjusted to $2x10^5$/ml and differentiation was induced by adding 1ml of cells to 1ml of IL-3 depleted medium containing G-CSF, (IMDM containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 ug/ml streptomycin and 10ng /ml of G-CSF, i.e 0.1ug or 10ul of a 10ug/ml solution). At 24 hour intervals, 10% of media was removed (200ul) and replaced with 200ul of fresh media containing G-CSF. Wright stained cytospin preparations were prepared at intervals and analysed for cellular morphology.

2.14 Flow cytometry

Cells were washed in PBS, 0.5% BSA and 0.05% sodium azide and pre-incubated with unlabelled anti-Fcγ III/II receptor mAb (2.4G2) for 15-30 minutes prior to staining with the primary antibody. Each subsequent stain was performed for 30 minutes, following which the cells were washed with PBS, 0.05% sodium azide. Cells were stained with specific conjugated monoclonal antibodies and isotype control antibodies. Antibodies used for flow cytometry are shown in Table 5. Flow cytometry was performed using a Beckman Coulter Epics XL analyzer or a Cyan ADP analyser and data analysed using EXPO3 software or Summit 4.1 software (DakoCytomation) respectively.

Table 5 Antibodies used for flow cytometry

Antibody	Clone	Isotype	Supplier	Working dilution
Anti- Fcγ III/II	2.4G2	$IgG_{2b\,\kappa}$	BD Pharmingen	1:100
Anti-cKit PE	**2B8**	**$IgG_{2b\,\kappa}$**	**BD Pharmingen**	**1:100**
Anti-cKit FITC	2B8	$IgG_{2b\,\kappa}$	BD Pharmingen	1:100
Anti-Mac1 PE	**M1/70**	**$IgG_{2b\,\kappa}$**	**BD Pharmingen**	**1:100**
Anti-Mac1 FITC	M1/70	$IgG_{2b\,\kappa}$	eBioscience	1:100
Anti-Gr1 PE	**RB6-8CS**	**$IgG_{2b\,\kappa}$**	**BD Pharmingen**	**1:200**
Anti-Gr1 APC	RB6-8CS	$IgG_{2b\,\kappa}$	eBioscience	1:100
Anti-Ter119 PE	**Ter119**	**$IgG_{2b\,\kappa}$**	**BD Pharmingen**	**1:100**
Anti-F4/80 PE	F4/80	$IgG_{2a\,\kappa}$	Caltag	1:100
Anti-B220 PE	**RA3-6B2**	**$IgG_{2a\,\kappa}$**	**BD Pharmingen**	**1:100**
Anti-CD41-PE	PEG30	$IgG_{2a\,\kappa}$	Research Diagnostic	1:200
Anti-CD61-FITC	**2C9.G3**	**$IgG_{2a\,\kappa}$**	**eBioscience**	**1:200**
Anti-IgG2A PE	G155-178	$IgG_{2a\,\kappa}$	BD Pharmingen	1:100
Anti-IgG2B PE	**MPC-11**	**$IgG_{2b\,\kappa}$**	**BD Pharmingen**	**1:100**
Streptavidin PE	N/A	N/A	BD Pharmingen	1:250

Mac1 is a member of the integrin subfamily comprising an alpha subunit (CD11b) and beta-2 subunit (CD18), and the two covalently associate to form Mac-1 which binds to CD54 (ICAM-1), C3b and fibrinogen. Mac1 is expressed on myeloid cells, natural killer (NK), activated T cells and a B cell subset, and upregulation of Mac1 is particularly seen with leucocyte activation.

Integrin CD41 (a2b), and integrin CD61 (beta 3) associate to form the heterodimeric integrin a2b-beta3 (CD41/CD61) which is a fibronectin

expressed in platelets. However, CD41 has also been implicated as a target of the SCL/TAL1 transcription factor to define the divergence of haematopoietic cells from endothelial cells at the earliest stages of haematopoietic development in the mouse embryo (Mikkola, *et al* 2003a). Thus CD41 characterises early murine haematopoietic progenitors and there is subsequent down regulation by the foetal liver stage.

The F4/80 antibody recognizes a member of a family of genes including human epidermal growth factor (*EGF*) module-containing mucin-like hormone receptor 1 and human *CD97* (Hume, *et al* 2002). The function of F4/80 is not clear but F4/80 antigen is present on the cell surface of a family of cells that includes all well-defined members of the mouse monocyte-phagocyte system (MPS). F4/80 protein expression is positively correlated with macrophage maturation and the antigen has often been used as a 'pan-macrophage' marker.

The cell surface receptor Gr1 (CD97) is a member of the G protein coupled receptor family and acts as a receptor potentially involved in both adhesion and signalling processes early after leucocyte activation, binding to Decay Accelerating Factor, a member of the CCP superfamily. It is expressed by the myeloid lineage in a developmentally regulated manner in the bone marrow. The expression of Gr1 on bone marrow granulocytes as well as on peripheral neutrophils is a good marker for these populations. Gr1 is known to be upregulated upon neutrophil differentiation of 32Dcl3 cells (Bigas, *et al* 1998).

2.15 MTS assay

Cells were seeded at a density of between 10^3 and 10^4 cells per well of a flat bottomed 96-well plate. After a specific period of culture, CellTiter 96® Aqueous One Solution reagent (Promega) was added to each well according

to manufactuer's instructions. The plates were wrapped in foil and returned to culture for 4 hours. After this time the cell viability was measured by reading the absorbance at 490nm using a 550 BioRad plate-reader.

2.16 Isolation of total RNA

TRIzol®Reagent (Invitrogen) was used to lyse cells and extract total RNA. Cells were harvested in conditions avoiding RNAse contamination and lysed in 1ml of TRIzol® per 10^7 cells and either stored at -80°C for later extraction, or left for 10 minutes at room temperature prior to immediate extraction. 0.2ml of chloroform per 1ml TRIzol® was added to each sample, vigorously mixed and centrifuged at 12 000 x g for 15 minutes at 4 °C. The upper layer was carefully transferred to a fresh tube without disturbing the DNA interface and 0.5ml of isopropanol per 1ml TRIzol® added. RNA was precipitated at room temperature for 10 minutes, and then pelleted at 12 000 x g for 10 minutes at 4°C. The RNA pellets were washed in 1ml of 70% ethanol and centrifuged at 7500 x g for 5 minutes at 4°C. The pellets were air dried before being dissolved in diethyl pyrocarbonate (DEPC)-treated water (Invitrogen) and stored at -80°C until used.

2.17 RT-PCR for cDNA synthesis

cDNA was synthesised from total RNA for further quantification of transcripts using quantitative PCR (Q-PCR). Reactions were carried out in 40ul reaction volumes. RNA was initially denatured in a 20ul reaction volume containing 10ug total RNA, 2ul DNAse enzyme, 2ul DNAse enzyme buffer, and DEPC-treated water to a total volume of 18ul and was incubated at room temperature for 15 minutes. A further 2ul of 25mM EDTA was added to terminate the reaction, then incubated at 65 °C for 5 minutes then placed on ice. To this was added 20ul of RT reaction containing 8ul first Strand buffer (x 5), 4ul DTT, 2ul random hexamers (0.32ug/ul) (Sigma-

Aldrich), 2ul deoxynucleotide triphosphate (dNTP) (10mM for each dNTP), 2ul RNase Out and 2ul MMLV-Reverse transcriptase (Invitrogen). The cDNA synthesis reaction was carried out in a PCR machine. 1 cycle was performed which consisted of 37 °C for 2 hours then 70 °C for 15 minutes. cDNA diluted with DEPC-treated water to obtain a concentration of 50ng/ul and stored at -20 °C.

2.18 Microarray analysis of gene expression

Three 32DMAF9 mixed populations were seeded at 2 x 10^5/ml in fresh medium for 12 hours prior to culturing with or without doxycycline for 24 hours, and eight 32DMENL clones seeded to 2 x 10^5/ml in fresh medium also. Experiments were carried out in triplicate to compensate for biological and experimental variation and performed according to the schema outlined in Figure 4.3 and Figure 4.4..

Cell culture conditions, reagents and sample processing were kept identical for all samples. Western blotting analysis was used to confirm the inducible expression of MLL-AF9, and constitutive expression of MLL-ENL. Samples were prepared according to Affymetrix Gene Expression Technical Manual using the Microarray cDNA synthesis kit 3315622 (Roche). An overview of miroarray technology is shown in Figure 2.2. Total RNA was isolated using TRIzol reagent and further purified. The quality and quantity of total RNA was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies) such that the A260/A280 ratio was at least 1.8, and the Bioanalyser gel profile exhibited a 28S band twice the intensity of the 18S ribosomal RNA. 15ug of total RNA were used to synthesise single stranded cDNA by reverse transcription using a T7-Oligo(dT) promoter primer in the first-strand synthesis reaction. The second strand cDNA synthesis reaction was carried out following removal of hybridised RNA using RNAse H, and the double-stranded cDNA was purified. This was then used as a template for the *in*

vitro transcription (IVT) reaction in the presence of T7 RNA polymerase and biotinylated UTP and CTP to produce biotin-labelled cRNA from the double stranded cDNA. The quality and quantity of cRNA was determined using an Agilent 2100 Bioanalyzer. The cRNA was then fragmented in the presence of heat and Mg^{2+} before hybridisation to the test array for assessment of target quality and labelling efficiency. The sample was then hybridised to the standard Affymetrix GeneChip® Mouse Genome 430 (version 2) array for 16 hours at 45°C, washed and stained with streptavidin-phycoerythrin (SAPE). The fluorescence was amplified by adding a biotinylated anti-streptavidin antibody and more SAPE, and the arrays scanned following excitation at 570nm using a GeneChip® Scanner 30000 (Affymetrix).

For MLL-AF9 experiments, one probe array was used for cells grown in the absence of doxycycline (control) and one for cells grown in the presence of doxycycline. For MLL-ENL experiments, one probe array was used for cells lacking expression of MLL-ENL and one probe array used for cells expressing MLL-ENL.

Affymetrix Microarray Suite 5.0 software was used to calculate gene expression values for each probe array. Data was analysed using GeneSpring 7.2 software. Each array underwent normalisation procedures to allow comparison of gene signals across multiple arrays. For MLL-AF9 experiments, gene expression of the induced sample was normalised to the non-induced control sample for each experiment. For MLL-ENL experiments, gene expression of the non-expressing sample was normalised to the MLL-ENL expressing sample. Data was subsequently filtered to be reliably detected in both the control and experimental samples. These genes were further filtered by Welch t-test to identify genes which were statistically significantly changed by greater than 1.5 fold by the expression of MLL-AF9 or MLL-ENL. Multiple testing correction on this set of genes was performed and using the Benjamini-Hochberg false discovery rate procedure applied.

Microarray technology was used to analyse the effect of MLL-ENL and MLL-AF9 on mRNA transcripts on a genome wide scale. Affymetrix GeneChip® Mouse Genome 430 (version 2) microarrays were used containing a series of eleven 25-mer probes per transcript paired with eleven mismatched probes to control for hybridisation specificity for each transcript, and assess performance of the probes. Probe set algorithms using Affymetrix Microarray Suite 5.0 software were used to interpret the signals from each probe set and generate a single signal per probe set. By comparing the level of hybridisation of the perfect match and the corresponding mismatch probes, a detection call of present or absent was assigned for each gene.

A detection algorithm was used to generate a detection *p* value reflecting the confidence of the detection call for each probe set containing multiple probe pairs. Subtraction of the mismatch probe value, to account for non-specific binding, resulted in a signal value for each gene. This gene signal was then normalised to the entire microarray to allow comparison of the signal across multiple arrays. This was done by generating a scaling factor required to adjust the total average signal of each array to an arbitrary target of 100. The scaling factor was applied based on the constitutively expressing house-keeping genes and ensured that the expression levels in the experimental samples were comparable to the control. The scaling factor of each array was within 3 standard deviations of the mean, and the signal values for each array were then multiplied by the appropriate scaling factor. GeneSpring 7.2 software (Agilent) was used to perform statistical analysis to determine genes which were outliers and so given a low significance. Unequal variance was assumed between the experimental and control populations and the Welch t-test employed to measure standard deviation and detect genes significantly changed with a *p* value of less than 0.05. In order to reduce the chance of one false positive gene among those found to be significantly changed, a Bonferroni multiple testing correction was applied to the data using the Benjamini-Hochberg false discovery rate procedure.

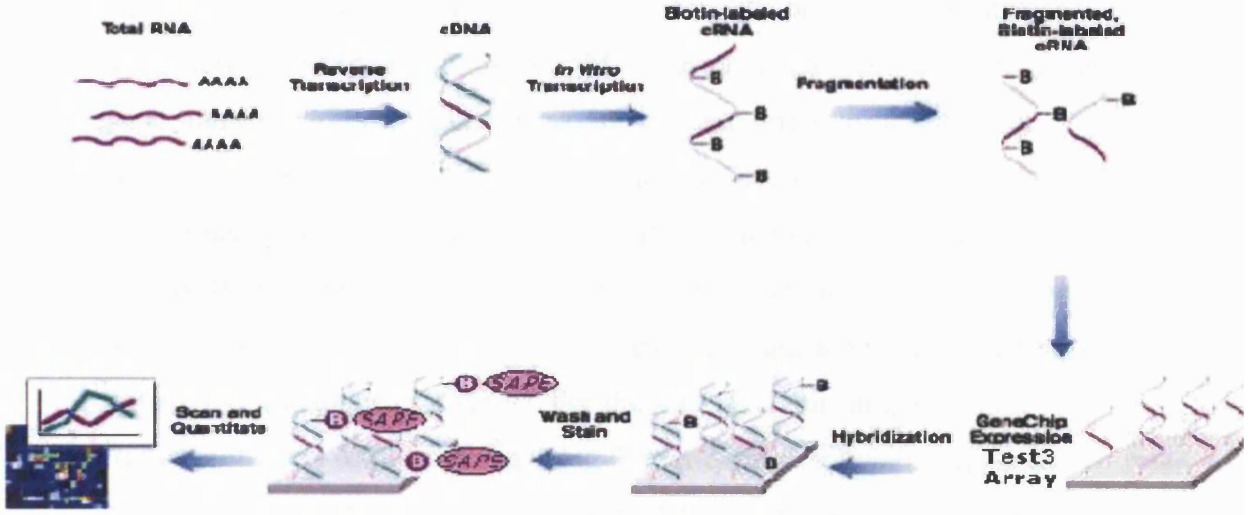


Figure 2.2 Overview of Microarray Technology.

Total RNA from the control and experimental populations is isolated and used to synthesise single stranded cDNA by reverse transcription. The second strand cDNA synthesis reaction is carried out following removal of hybridised RNA and the double-stranded cDNA purified. These are used as a template for the *in vitro* transcription (IVT) reaction in the presence of T7 RNA polymerase and biotinylated UTP and CTP to produce biotin-labelled cRNA from the double stranded cDNA. The cRNA are then fragmented before hybridisation to the Affymetrix GeneChip® Mouse Genome 430 array for 16 hours at 45°C, washed and stained with streptavidin-phycoerythrin (SAPE). The fluorescence is amplified by adding a biotinylated anti-streptavidin antibody and more SAPE, and the arrays scanned following excitation at 570nm using a GeneChip® Scanner 30000 (Affymetrix).

2.19 Q-PCR

PCR products were quantified to validate the results of microarray analyses and quantify the expression levels of MLL fusion genes. For candidate target genes and *Gapdh* control gene, Assay-on demand primer/probe sets and Taqman mastermix containing UNG (Uracil-N-Glycosylase to destroy contaminating products from previous Q-PCR reactions) were obtained from Applied Biosystems, USA. For each primer/probe set the 5' reporter of the probe was 6-carboxyfluorescein (FAM) and the 3' quencher was 6-carboxy-tetramethylrhodamine (TAMRA). For the *MLL-ENL* fusion gene, primer/probe sets were designed using Primer express software (Applied Biosystems). In order to measure *MLL-ENL* transcript expression by Q-PCR, two primers and a probe were designed by Dr. Sarah Horton (MHCB), using Primer express software (Applied Biosystems). The primers flanked the *MLL-ENL* breakpoint and the probe was designed such that it spanned the breakpoint:

Forward primer: 5' CAGGGTGGTTTGCTTTCTCTGT 3'
Reverse primer: 5' GCGATGCCCCAGCTCTAA 3'
Probe: 5' TGGACGGTGCACTCTACATGCCCACTA 3'

These sequences were submitted to Applied Biosystems who then synthesised the primers and probe. The 5' reporter of the probe was 6-carboxyfluorescein (FAM) and the 3' quencher was 6-carboxy-tetramethylrhodamine (TAMRA). The optimum primer and probe concentrations were determined by Dr. Sarah Horton (MHCB).

Taqman probes were were based on Fluorescent Resonance Energy Transfer (FRET) to generate a fluorescent signal in real time Q-PCR when used in a 5' nuclease assay that utilised the 5' to 3' exonuclease activity of Taq DNA polymerase. The Taqman mastermix + UNG containing 5' exonuclease

activity would release FAM from the probe when the gene of interest was correctly transcribed. Once cleaved from the rest of the probe the 5' dye molecule was freed from the quenching effect (via FRET) when in close proximity to the quenching molecule TAMRA. The amount of fluorescence reflected the quantity of gene present and was analysed using Sequence Detection System, version 1.2 software. 20ul reactions were set up in quadruplicate in a 96-well plate (ABS, USA) using 19ul of Taqman mastermix, primer/probe set for the gene of interest and DEPC-treated water, and 1ul (50ng) of sample cDNA. Parallel reactions were set up as internal controls using primer/probe sets for *Gapdh*. The plate of PCR reactions was placed in a in a Q-PCR 7000 machine and cycling conditions were 2 minutes at 50°C hold (UNG activation), 10 minutes 95 °C hold (UNG denatured), then 40 cycles of 15 seconds at 95°C (denaturing), 1 minute at 60 °C (annealing and extension).

Relative quantitation was carried out to compare the gene of interest with *Gapdh* within each cDNA sample. Quantitation was carried out relative to *Gapdh* by subtracting the cycle threshold (Ct) of this gene from the Ct of the gene of interest to give the difference in cycle number (ΔCt) for each sample studied.

Equation 1: ΔCt (sample) =Ct (gene of interest) – Ct (*Gapdh*)

The difference between the cycle numbers of the control population 32DtTS (ΔCt Calib) and the experimental 32DMENL samples was calculated as the $\Delta\Delta Ct$ for each sample.

Equation 2: $\Delta\Delta Ct = \Delta Ct$ (experimental sample) – ΔCt (Calibration sample)

The fold difference of template for the candidate and reference genes, studied in the experimental sample (32DMENL) compared to the calibration sample

(32DtTS), was the exponent of the base 2 due to the doubling function of PCR, and is represented by Equation 3.

Equation 3: $2^{-\Delta\Delta Ct}$

In a collaboration with the Haematology Department, CCRCB (Queen's University, Belfast), the global *Hox* gene expression profile of MLL-ENL immortalised cells was analysed by Q-PCR. The murine *Hox* genes were cloned and standard curves were generated for 38 of the 39 genes and for the Hox co-factors *Meis1* and *Pbx1*. These curves were used to convert Q-PCR data, which had been normalised to 18S ribosomal RNA expression, into *Hox* gene copy number.

2.20 Lentiviral shRNA constructs

The lentiviral constructs used in this study are shown in Figure 2.3.

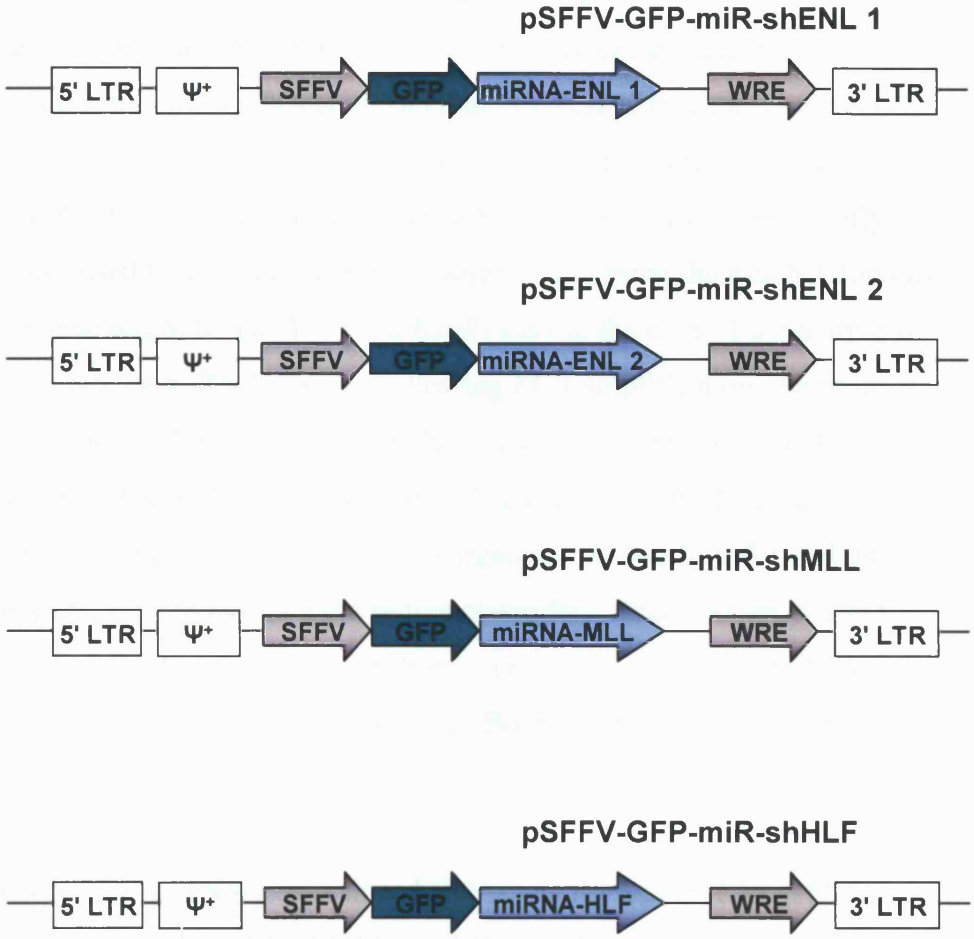


Figure 2.3 A diagram of the lentiviral shRNA constructs used in this study.

LTR: long terminal repeat, SFFV: spleen focus forming virus, ψ^+: viral packaging signal, WRE: Woodchuck hepatitis virus posttranscriptional regulatory element, GFP: green gluorescent protein.

shRNAs against a gene of interest were expressed using microRNA (miRNA) expression in pSFFV-GFP lentiviral vectors. Synthetic sense and antisense short hairpin oligoribonucleotides were designed using a web-based algorithm by Dr. Jasper de Boer and expressed in a lentiviral vector using the miR30-shRNA cloning protocol for the generation of pRIME (Potent RNA interference using MicroRNA expression) vectors. Briefly, PCR was used to amplify the 97mer hairpin oligo using the miRNA forward and reverse oligos to add *Xho*I and *EcoR*I sites to the ends of the hairpin for cloning into the miRNA vector. Following PCR amplification, the products were purified and both the PCR product (insert) and the vector (miRNA vector) digested with *Xho*I and *EcoR*I. The digested amplified hairpin inserts and miRNA vector were purified. The ligation mix was transformed into a strain of E.coli and clones were sequence-verified. Transfection of 293T packaging cells to produce concentrated lentivirus expressing miRNA-shRNA was carried out by Dr. Jasper de Boer according to standard procedures.

Sequences for miRNA expressing shRNAs against human *ENL* (shENL sequences 1 and 2), *MLL* (shMLL) and *HLF* (shHLF) genes are shown in Table 6.

Table 6 Sequences for miRNA constructs used

Oligo name	Sequence
shENL 1	TGC TGT TGA CAG TGA GCG CGC GAG AAG CTC ACC T TC AAC ATA GTG AAG CCA CAG ATG TAT GTT GAA GG T GAG CTT CTC GCA TGC CTA CTG CCT CGG A
shENL 2	TGC TGT TGA CAG TGA GCG AGC AAA GAC TCC GAG A GC AAG ATA GTG AAG CCA CAG ATG TAT CTT GCT CT C GGA GTC TTT GCG TGC CTA CTG CCT CGG A
shMLL	TGC TGT TGA CAG TGA GCG CGG AGA TAA GAT CAA G AA GAA ATA GTG AAG CCA CAG ATG TAT TTC TTC TT G ATC TTA TCT CCA TGC CTA CTG CCT CGG A
shHLF	TGC TGT TGA CAG TGA GCG CGC AAG AAC ATA CTT G CC AAG TTA GTG AAG CCA CAG ATG TAA CTT GGC AA G TAT GTT CTT GCA TGC CTA CTG CCT CGG A

A series of lentiviral vectors have been developed that provided high penetrance regulatable knockdown at single copy (pPRIME), and allow for the tracking of shRNA expression with a variety of reporter genes (Stegmeier, *et al* 2005). A system of pPRIME vector delivery of miRNA embedded shRNA was used to downregulate the expression of *MLL-ENL* in 32DMENL cells. In addition, to enhance the efficiency of expression of the shRNA transduction and expression, the pRIME vector was modified to express the miRNA from the U3 part of the SFFV strain P LTR sequence (SFFV-U3LTR). The GFP was expressed upstream of the WPRE and efficiency of lentiviral infection was assessed by flow cytometric analysis of GFP.

2.21 Transduction of 32Dcl3 cell lines with lentiviral shRNA constructs

32Dcl3 cell lines were transduced at a density of 10^4 cells per well of a 96-well flat-bottomed plate. A total of 6 wells were set up for each condition and 6×10^4 of control and 32DMENL cells were transduced with a total of 600 µl

lentivirus. The viral supernatant was supplemented with 20% FCS, 50 μM 2-mercaptoethanol (2-ME) (BDH), 5 μg/ml polybrene, and 10 ng/ml interleukin-3 (IL-3). All recombinant murine growth factors were supplied by Peprotech EC. The cells were transduced by spinoculation (centrifugation at 700 g, 25°C, 45 minutes) and then returned to culture. Cell culture conditions were optimised by the addition of a supplement of 10ng/ml IL-3 to the culture medium after 24 hours. The transduced cell lines were kept in culture and expanded within 4 days to allow harvesting of 1 x 10^5 cells for FACS analysis of GFP expression, and 1 x 10^6 cells for Western analysis of MLL-ENL expression.

2.22 Retroviral constructs

Diagrams of all the retroviral constructs are shown in Figure 2.4. All of the MLL-fusions were sequenced prior to sub-cloning by Dr. Dale Moulding and Dr. Inusha de Silva of the Molecular Haematology and Cancer Biology (MHCB) unit and sub-cloning carried out by Dr. Sarah Horton of the Molecular Haematology and Cancer Biology (MHCB) unit. Comprehensive restriction digests of the constructs were performed by Dr. Sarah Horton and DNA sequencing around the cloning sites of the MLL fusion constructs confirmed that errors had not been introduced during the sub-cloning process. The pMSCV-Neo-MLL-ENL$^{\Delta 1\text{-}26}$ construct was made by Dr. Sarah Horton (MHCB) and lacked the first 26 amino acids of MLL containing the high-affinity Menin-binding domain. The pMSCV-Neo-MLL-ENL$^{\text{full-length}}$ construct was made by Dr. Inusha de Silva (MHCB) and retained this domain. The pMSCV-Neo-E2A-HLF construct was made by Dr. Jenny Yeung (MHCB).

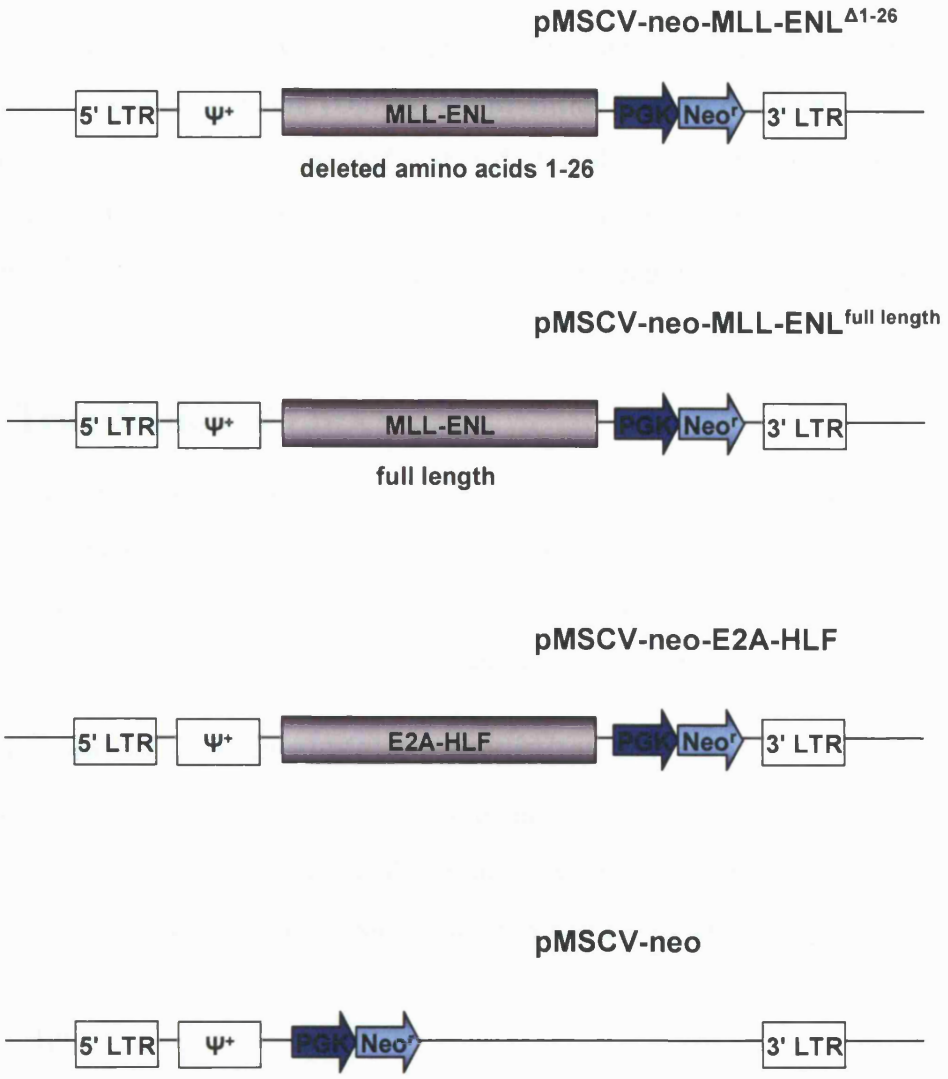


Figure 2.4 A diagram of fusion gene retroviral constructs used in this study.

LTR: long terminal repeat, ψ^+: viral packaging signal, PGK: phosphoglycerate kinase promoter, Neo^r: neomycin resistance gene.

2.23 Culture of LinXE and 293T cell lines

The LinXE ecotropic retrovirus packaging cell line and 293T cell lines were cultured in complete medium (Dulbecco's Modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% foetal calf serum (FCS) and 2 mM L-glutamine). The LinXE cells were maintained in 7.5 μg/ml hygromycin (Cayla) to select for the presence of the gag, pol and env genes.

2.24 Transfection of LinXE cells

LinXE cells were seeded at a density of 0.75 x 10^6 per 10cm plate 3 days prior to transfection. Cells were transfected with 8 μg retroviral plasmid DNA using 40 μl Lipofectamine (Invitrogen) in a total volume of 8 ml optimum (Invitrogen) according to the manufacturer's instructions.

2.24.1 Isolation of haematopoietic progenitor cells

HPCs were isolated from mice which were maintained in the animal facilities of the National Institute for Medical Research. All experiments were performed according to institutional guidelines and Home Office regulations.

Foetal HPC

Ter-119$^-$ HPCs were purified from embryonic day 13.5 (E13.5) foetal liver of mice by HPCs were purified by magnetic activated cell sorting (MACS) using MACS separation columns (Miltenyi Biotech) and monoclonal antibodies specific to Ter119 .

Adult HPC

Bone marrow was extracted from 4-6 week old mice using standard techniques. A single cell suspension was made from the marrow of the femur and tibia and the red cells were lysed in 2 ml of erythrocyte lysis buffer for 10 minutes at room temperature. HPCs were purified by MACS using MACS

separation columns (Miltenyi Biotech). Lineage depleted cells were isolated by MACS using a lineage panel kit which consisted of anti-CD3 (145-2C11), anti-Mac1 (M1/70), anti-B220 (RA3-6B2), anti-Gr1 (RB6-8C5), anti-Ter-119 (BD Pharmingen).

2.24.2 Culture of HPC

Foetal HPCs were seeded at a density of 2 x 10^5/ml and adult HPCs were seeded at a density of 1 x 10^6/ml. HPCs were stimulated overnight in complete medium supplemented with 50 μM 2-ME (BDH), 100 ng/ml stem cell factor (SCF), 10 ng/ml interleukin-6 (IL-6) and 10 ng/ml IL-3. All recombinant murine growth factors were supplied by Peprotech EC.

2.25 Transduction of haematopietic progenitor cells with retroviral constructs

Retroviral supernatant was harvested as described previously. HPCs were transduced on two consecutive days. Complete medium was added to the LinXE cells after the first virus harvest and fresh virus was collected from the same culture the following day. The retroviral supernatant was concentrated 10-fold in some experiments. The supernatant was cleared of cell debris by 2 rounds of centrifugation at 580g for 5 minutes. Cleared virus was then aliquotted into 1.5ml eppendorf tubes and concentrated by centrifugation for 1 hour at 16,000g. HPCs were transduced at a density of 10^4 cells per well of a 96-well flat-bottomed plate. A total of 6 wells were set up for each condition and 6 x 10^4 HPCs were transduced with a total of 600 μl virus unless otherwise stated. The viral supernatant was supplemented with 20% FCS, 50 μM 2-ME, 5 μg/ml polybrene, 100 ng/ml SCF, 10 ng/ml IL-3 and 10 ng/ml IL-6. The cells were transduced by spinoculation (centrifugation at 700 g, 25°C, 45 minutes) and then returned to culture. The transduction was repeated after 24 hours, 60 μl of medium was removed

from each well and replaced with 100 μl of fresh viral supernatant and growth factors. After culture overnight, the transduced HPCs were used for colony forming assays.

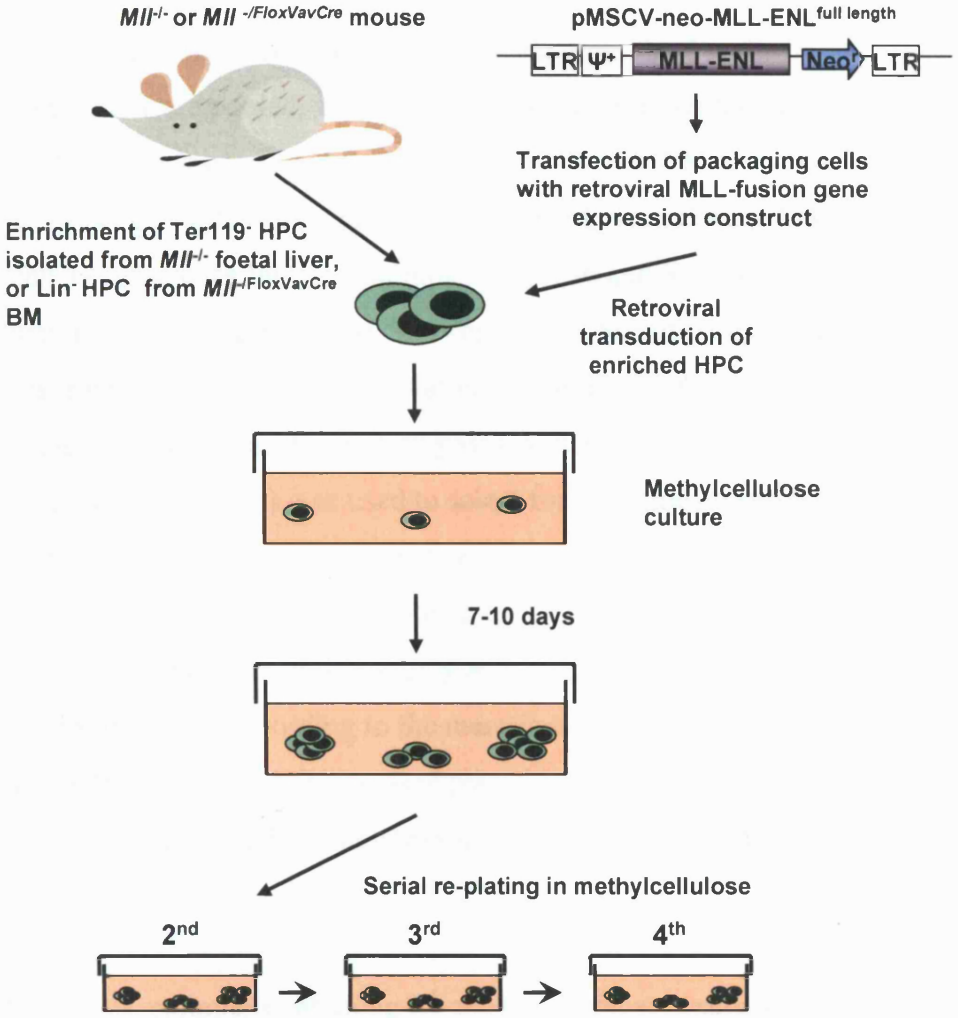


Figure 2.5 Experimental strategy of haematopoietic progenitor cell retroviral transduction.

See text for details.

2.26 Colony forming assays

Approximately 24 hours after the second transduction, the 6 wells of transduced cells were pooled, washed in 5 ml Minimal Essential-Alpha Media (MEM) (Invitrogen) and resuspended in 100 µl MEM. For the myeloid assays the cells were added to 3 ml of methylcellulose (M3434) (Stem cell Technologies Inc.), containing IL-3, IL-6 and SCF which was supplemented with 10 ng/ml GM-CSF (Peprotech). Selection was employed in the first round of methylcellulose plating. 1 mg/ml G418 (Invitrogen) was used to select for cells transduced with pMSCV-neo constructs and 1.5 mg/ml hygromycin (Cayla) was used to select for cells transduced with pMSCV constructs. The volume of methylcellulose containing the cells, antibiotic and growth factors was made up to 3.3 ml with MEM. The components were then mixed thoroughly and 1.1 ml was aliquotted into duplicate 35mm plates according to the manufacturer's instructions. The duplicate methylcellulose cultures were placed in 10cm plates and cultured for 6-10 days alongside a 35mm plate containing PBS to humidify the cultures.

After 6-10 days, colonies containing 50 cells or more were scored. The cells were harvested from the methylcellulose by the addition of 1 ml MEM to each plate, followed by gentle pipetting to create a single cell suspension. The cells from duplicate methylcellulose cultures were pooled and washed in 10 ml MEM containing 2% FCS. The cells were counted and 1 x 10^4 cells were replated into secondary assays under identical conditions but in the absence of antibiotic selection. This process was repeated every 7-10 days. Colonies were stained with 1 mg/ml p-iodonitrotetrazolium (INT) (Sigma-Aldrich) in PBS.

2.27 PCR for genotyping

DNA samples for genotyping of mice were prepared from unsorted foetal liver or BM cells using the hot sodium hydroxide and tris method (see HotSHOT genomic DNA preparation, Camper Lab, Biotechniques 2000 29 (1) 52-54). Briefly 1 x 10^5 cells were washed in PBS, spun at 4°C at 4000rpm for 5 minutes. The cells were lysed in 25ul of HotSHOT alkaline lysis reagent at 95°C for 20 minutes, then immediately neutralized with 25ul of HotSHOT neutralization buffer. 2ul of DNA was used in a 25ul PCR reaction. DNA samples for genotyping of individual colonies grown in methylcellulose were prepared by picking individual colonies into 100ul of PBS each and washing twice with PBS. 25ul of 0.05M sodium hydroxide were added to each sample and boiled for 5 minutes. 2.5ul of 2M Tris pH 7.5 were added to each sample and spun at 13 000 rpm for 1 minute. 4ul of DNA were used in a 25ul PCR reaction.

The DNA was analysed by PCR using three combinations of primers. The primers and their binding sites in the different alleles are shown in Figure 5.6. PCR reactions for genotyping of the *Mll* gene were carried out using the Advantage GC2 PCR kit according to manufacturer instructions (BD-Biosciences). The primer combinations are shown in Table 7. Genotyping to detect the 5' LoxP site used primers E and F, and primers E and G and the programme used was [97°C 3 min, (97°C 30s, 62°C 1 min, 72°C 2 min) x 3 cycles, (96°C 30s, 62°C 1 min, 72°C 2 min) x 7 cycles, (96°C 30s, 61°C 1 min, 72°C 2 min) x 10 cycles, (96°C 30s, 60°C 1 min, 72°C 2 min) x 10 cycles, 72°C 10 min].

Table 7 Primer combinations used for genotyping of the *Mll* allele

Primer name	Primer sequence
E	5' GCCAGTCAGTCCGAAAGTAC
	3'
F	5' AAGATGTTCAAAGTGCCTGC
	3'
G2	5' GCTCTAGAACTAGTGGATCCC
	3'

3 Expression of MLL-ENL in 32Dcl3 cells confers an altered phenotype

3.1 Introduction

MLL fusion proteins MLL-ENL and MLL-AF9 were expressed in 32Dcl3 cells and the phenotype of cells expressing MLL-ENL was studied.

32Dcl3 cells are a murine multipotential (erythroid-neutrophil-mast cell-basophil-eosinophil) cell line derived from long term bone marrow cultures of Toll-like receptor defective C3H/HeJ mice infected with the Friend murine leukaemia virus (Greenberger, *et al* 1983). This cell line was chosen for its characteristics as a non-transformed, immortalised murine myeloid precursor cell line which has been previously used to demonstrate pathways of haematopoietic differentiation in response to erythropoietin (EPO), granulocyte-macrophage colony stimulating factor (GM-CSF) and granulocyte colony stimulating factor (G-CSF) (Migliaccio, *et al* 1989). Of the MLL fusion proteins studied, MLL-AF9 is found almost exclusively in infant myeloid leukaemias, and MLL-ENL is found equally in myeloid and lymphoid infant leukaemias. This myeloid precursor cell line therefore appeared to be appropriate for the investigation of target genes of these fusion proteins in myeloid leukaemias, as well as for the study of the effects of these fusion proteins in terms of their effect on cellular differentiation and requirement of IL-3 for survival.

3.2 The tetracycline inducible system

The ideal conditional expression system should allow the ability to switch transgene expression on and off, rapidly and reversibly in a chosen cell type. The tetracycline transactivator system has been established as a reliable tool for regulated transgene expression, and exploits the pathway that controls the expression of a tetracycline resistance gene in *E. coli* (Gossen and Bujard

1993). 32Dcl3 clones which inducibly expressed a gene of interest were generated using the Tet-On gene expression system (Clontech) by which gene expression follows the addition of doxycycline, a tetracycline derivative.

The Tet repressor protein (TetR) is a regulatory protein which negatively regulates the expression of genes of the tetracycline resistance operon in *E. coli* by binding to Tet operator sequences (TetO) in the absence of tetracycline. In the presence of tetracycline, dissociation of the TetR normally occurs to allow expression of the tetracycline resistance protein (TetA). The regulatory protein used in the Tet-On system is the reverse tetracycline-controlled transactivator (rtTA) which is a transcriptional activator protein which responds to doxycycline. It is based on a 'reverse' Tet repressor protein (rTetR) which results from a four amino acid change in the TetR that reverses the protein's response to doxycycline,. The rtTA is comprised of the rTetR fused to the C-terminal 127 amino acids of the Herpes simplex virus VP16 activation domain, which converts the rTetR from a transcriptional repressor to a transcriptional activator (Gossen and Bujard 1992). The rtTA is located upstream of the *CMV* promoter (P CMV) in the pTet-On regulatory plasmid, which also contains a neomycin resistance gene.

The second component of the Tet-On system is the response plasmid pTRE which expresses a gene of interest under the control of the tetracycline-response element (TRE). The TRE consists of seven direct repeats of a 42-bp sequence containing the TetO, and is located just upstream of the minimal *CMV* promoter (Pmin CMV). This minimal promoter lacks the strong enhancer elements normally associated with the *CMV* immediate early promoter so that little or no background expression of the gene of interest should occur in the absence of binding of the rtTA to the TRE. In the Tet-On system, binding of rtTA to the TRE occurs only in the presence of

doxycycline as it causes a conformational change of the rtTA to allow binding to the TRE and activation of transcription of the gene of interest. Thus, transcription is turned on in response to doxycycline and in a dose-dependent manner (Gossen and Bujard 1995).

The control of background constitutive expression can additionally be optimised by the co-transfection of the pTet-tTS plasmid which expresses the tetracycline-controlled transcriptional silencer (tTS). This is a fusion of the Tet repressor protein (TetR) and the KRAB-AB domain of the KID-1 protein (Freundlieb, *et al* 1999, Witzgall, *et al* 1994), and prevents gene expression in the absence of doxycycline by binding to the TetO sequences in the TRE. The addition of doxycycline switches the system from one that is actively silenced by tTS to one that is induced by rtTA. The activation of doxycycline responsive gene transcription in the Tet-On system is illustrated in Figure 3.1.

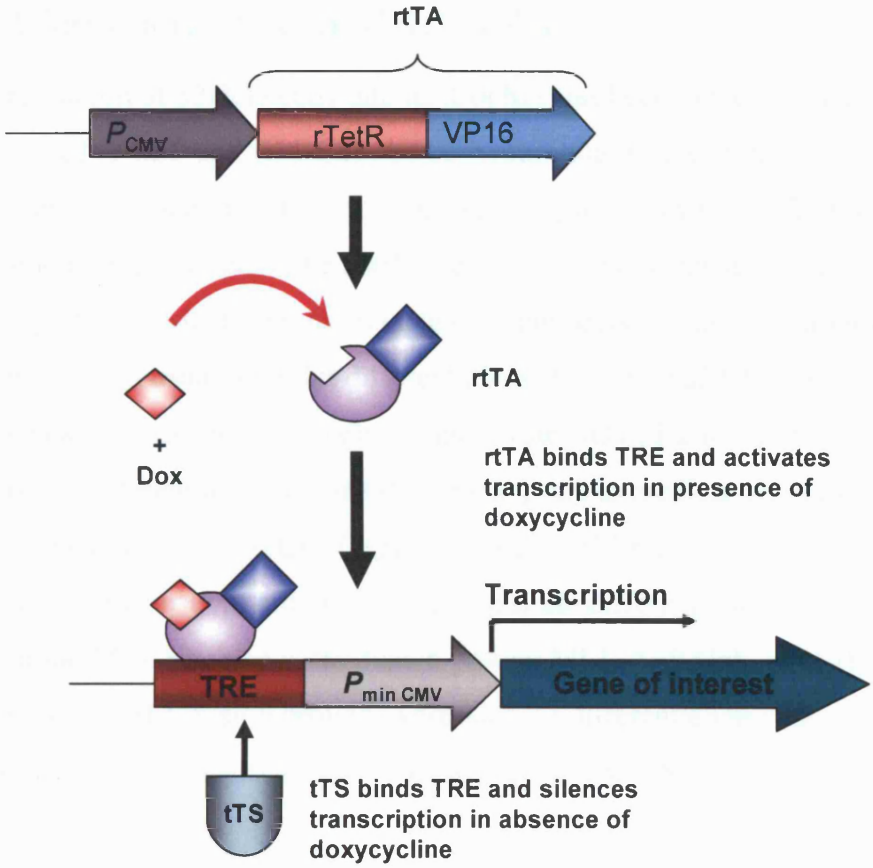


Figure 3.1 Regulation of gene expression by the Tet-On system.

The rtTA is a transcriptional activator protein which responds to doxycycline. Binding of rtTA to the TRE occurs only in the presence of doxycycline following a conformational change of the rtTA, to allow subsequent activation of transcription of a gene of interest. The tTS prevents gene expression in the absence of doxycycline by binding to Tet operator sequences in the TRE.

rTetR: reverse Tet repressor, VP16: VP16 activation domain, rtTA: reverse Tetracycline-controlled transactivator, P_{CMV}: *CMV* promoter, TRE: Tetracycline-response element, $P_{min\ CMV}$: minimal *CMV* promoter, tTS: Tetracycline-controlled transcriptional silencer..

3.3 Characterisation of 32Dcl3 cells

Differentiation of 32Dcl3 cells into neutrophils has been demonstrated in the presence of G-CSF and GM-CSF, by both morphological criteria and a well-characterised system of cell surface markers (Ingles-Esteve, *et al* 2001). This differentiation is thought to be partly mediated by the *Notch* gene family whose protein products are involved in cell fate decisions in haematopoietic progenitors and usually inhibit differentiation (Milner, *et al* 1996). G-CSF has been shown to phosphorylate and inactivate NOTCH2 to allow neutrophil differentiation, while GM-CSF appears to mediate differentiation through its effect on NOTCH1(Ingles-Esteve, *et al* 2001). 32Dcl3 cells have been used previously in one study to demonstrate differentiation arrest by the N-terminal MLL retained in the fusion protein MLL-AF9 (Joh, *et al* 1999). The effect of MLL fusion protein expression on differentiation was assayed in this study following exposure of 32Dcl3 cells to G-CSF.

A further characteristic of 32Dcl3 cells is their dependence on IL-3 for survival. DNA histograms have previously shown 40% of cells in G0/G1 phase and 40% in S phase when incubated with this growth factor (Shimada, *et al* 1993). These cells undergo significant apoptotic death following IL-3 withdrawal (Houben, *et al* 1997). Over-expression of oncogenes such as *Bcl2* has been shown to protect these cells from such apoptosis (Baffy, *et al* 1993). In this study the effects of MLL fusion proteins in conferring IL-3 independent survival, or increased sensitivity to apoptosis can be readily determined following withdrawal of this cytokine.

When maintained in IL-3, 32Dcl3 cells proliferate in suspension as round undifferentiated blasts, growing singly or in small clusters with a doubling time of less than 24 hours (Shaknovich, *et al* 1998). The morphology of these cells was characterised following examination of Giemsa-Wright stained cytospin preparations in which the cells were seen to have a single large,

relatively round nucleus and scant dark blue cytoplasm containing few granules (Figure 3.2B). The cells were incubated with G-CSF to induce neutrophil differentiation according to previously described methods (Bigas, *et al* 1998). Evidence of neutrophil differentiation was seen at day six, with complete differentiation of the majority of cells by day fourteen in line with previous reports (Ingles-Esteve, *et al* 2001, Miranda, *et al* 2002) and is shown in Figure 3.3A. Criteria for neutrophil differentiation included increased cytoplasmic to nuclear ratio, increased cytoplasmic granulation, increased intensity of eosin staining and increased nuclear segmentation. Figure 3.2A shows the cell surface marker profile of 32Dcl3 cells in which they express the macrophage marker F4/80, and do not express the granulocyte marker Gr1 or granulocyte/macrophage marker Mac1. These results are consistent with an early precursor with capacity for macrophage or granulocyte differentiation.

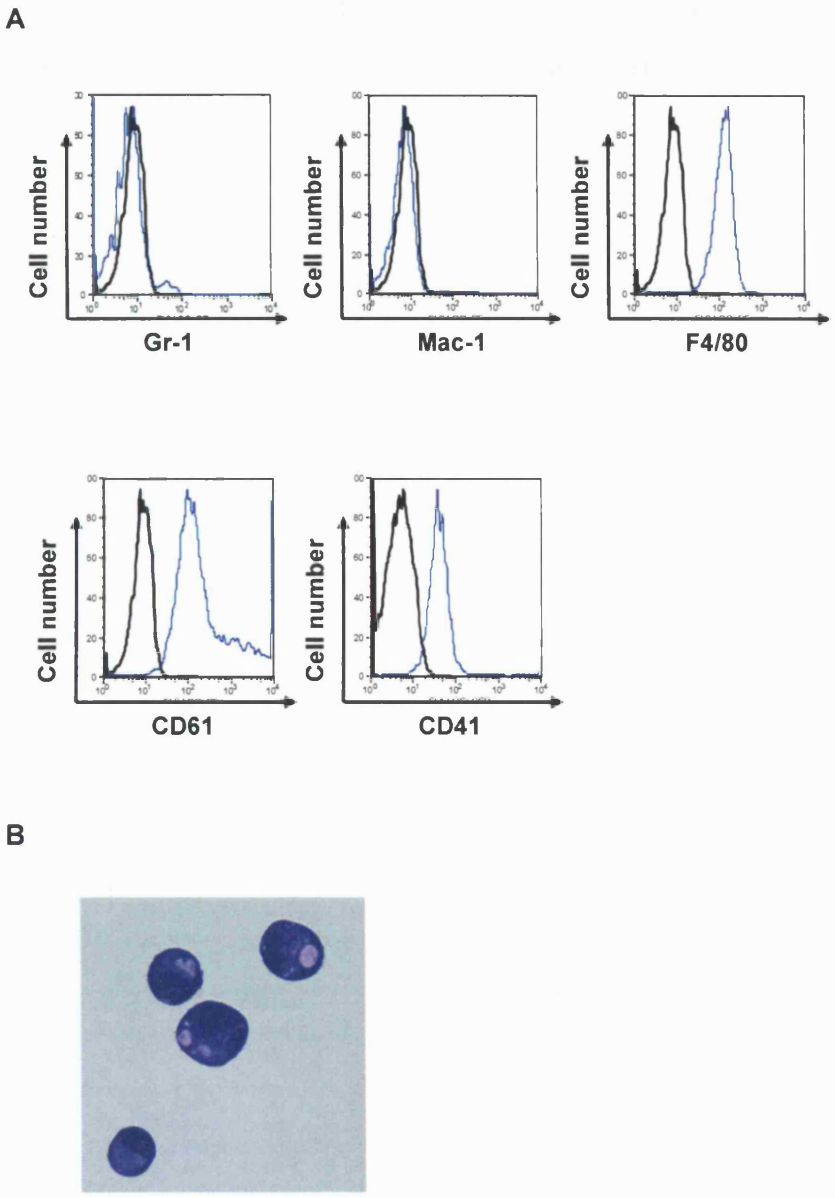


Figure 3.2 Characterisation of the phenotype of 32Dcl3 cells.

A Expression of cell surface markers Gr1, Mac1, F4/80, CD61 and CD41 by FACS analysis. Isotype control is shown in bold, and sample in blue.

B Cytospin Giemsa-stained preparation of 32Dcl3 cells.

In line with previous reports, the granulocyte marker Gr1 and granulocyte/macrophage marker Mac1 are up-regulated following G-CSF induced neutrophil differentiation as shown in Figure 3.3B.

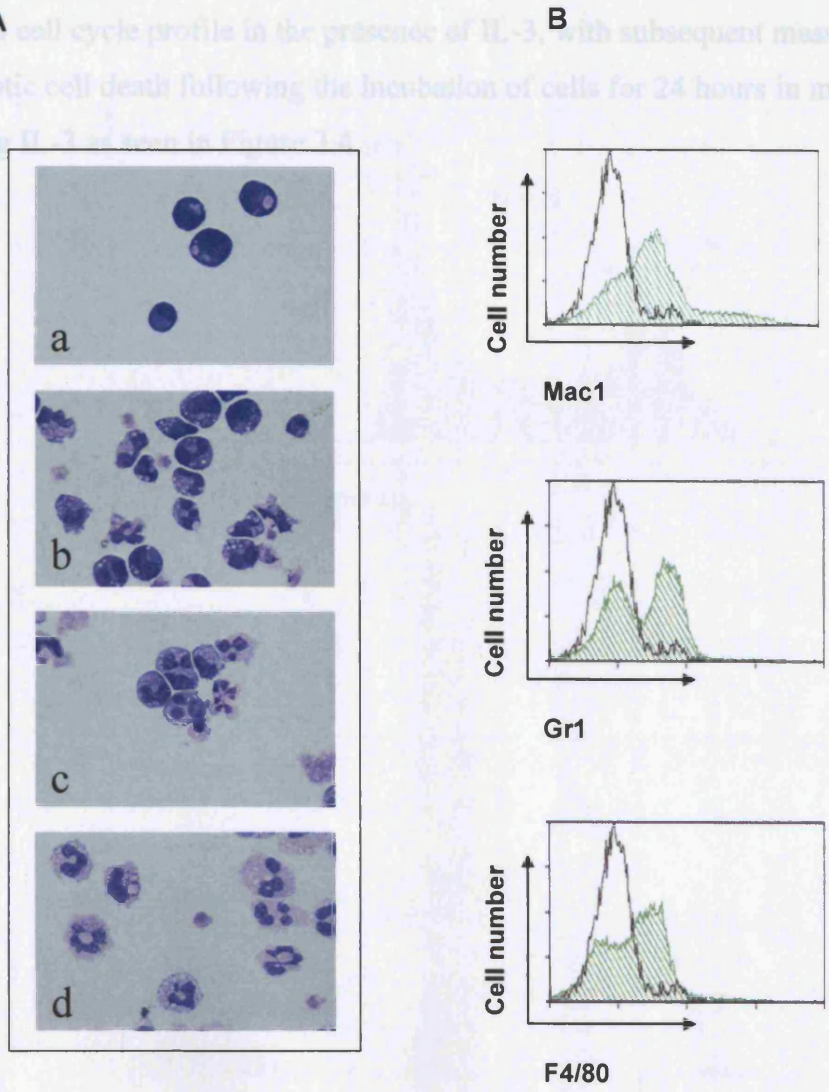
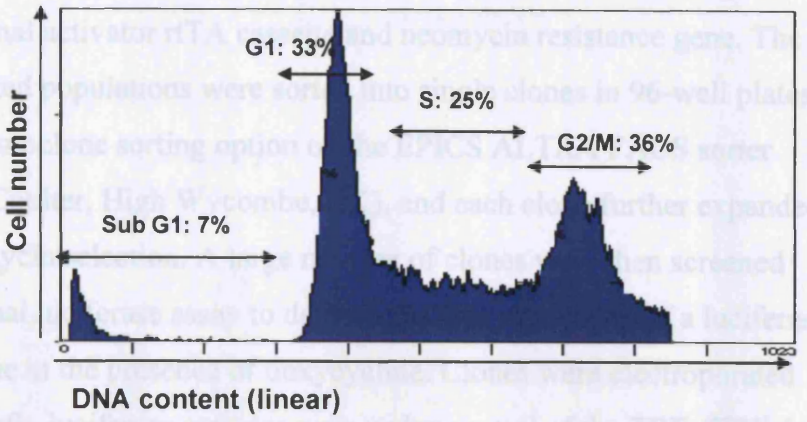


Figure 3.3 G-CSF mediated neutrophils differentiation of 32Dcl3 cells.
A Cytospin Giemsa-stained preparations of 32Dcl3 cells following incubation with G-CSF shows wild-type 32Dcl3 cells at day 0 (a), and subsequent neutrophil differentiation at day 6 (b), day 8 (c), and day 12 (d). **B** Expression of Gr1 and Mac1 cell surface markers at day 12 of differentiation. Isotype controls are in black, shaded areas represent samples.

Confirmation of IL-3 dependence of the 32Dcl3 cells was demonstrated by a normal cell cycle profile in the presence of IL-3, with subsequent massive apoptotic cell death following the incubation of cells for 24 hours in media lacking IL-3 as seen in Figure 3.4.

A



B

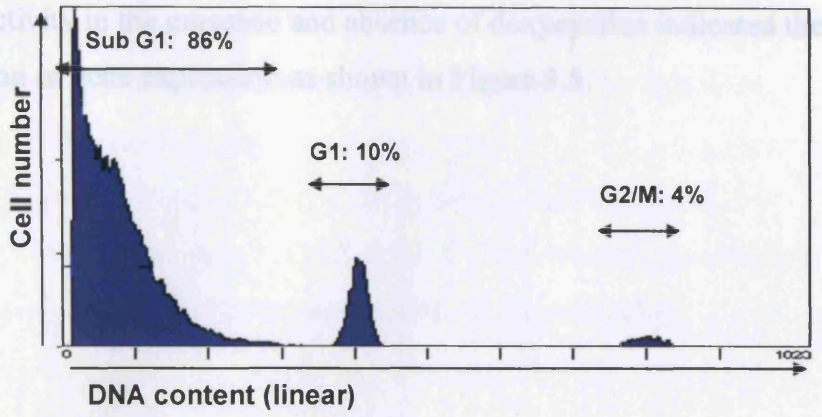


Figure 3.4 Cell cycle and apoptotic profile of 32Dcl3 cells.

A Cell cycle profile of 32Dcl3 cells when cultured with IL-3 shows the majority of cells in a phase of protein synthesis (G1), DNA synthesis (S), or DNA proof-reading and preparation for mitosis (G2/M).

B Cell cycle profile of 32Dcl3 cells following withdrawal of IL-3 from culture media for 24 hours shows the majority of cells having undergone apoptosis with associated DNA fragmentation (sub G1 phase)..

3.4 Generation of tetracycline inducible 32Dcl3 cells

32Dcl3 cells were electroporated with the TetOn plasmid containing the transcriptional activator rtTA cassette and neomycin resistance gene. The electroporated populations were sorted into single clones in 96-well plates using the Autoclone sorting option on the EPICS ALTRA FACS sorter (Beckman Coulter, High Wycombe, UK), and each clone further expanded under neomycin selection. A large number of clones were then screened using the dual luciferase assay to detect inducible expression of a luciferase reporter gene in the presence of doxycycline. Clones were electroporated with the firefly luciferase reporter gene under control of the TRE (TRE-Luc), together with the constitutively expressing *renilla* luciferase gene under control of the *CMV* promoter as a control for transfection efficiency. Cells were incubated with doxycycline for 24 hours. Comparison of firefly luciferase activity in the presence and absence of doxycycline indicated the fold induction of gene expression as shown in Figure 3.5.

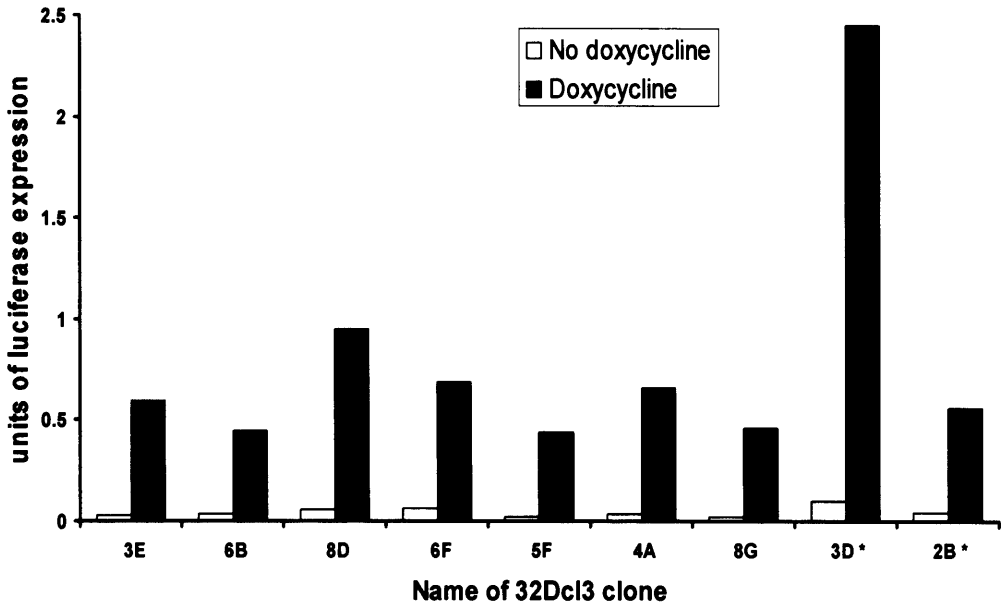


Figure 3.5 Inducible expression of luciferase reporter by 32Dcl3 tetracycline-responsive clones.

The degree of luciferase expression in the presence and absence of doxycycline is shown for each 32Dcl3 clone. (*Clones stably transfected with luciferase reporter constructs and selected in hygromycin).

In this way, doxycycline responsive inducible 32Dcl3 clones were generated that would express a gene of interest in a regulatable and temporal manner. Specific clones were chosen which had the lowest levels of background expression in the absence of doxycycline, together with the highest levels of gene induction. The two clones chosen were those named 3E and 8D, with 8D being chosen for its higher level of induction despite a higher level of background expression.

Isolating such clones involved an extensive screening process in which large numbers of clones were tested, and for this reason individual clones were not tested in triplicate. However, the clones 3E and 8D were used repeatedly and inducubility was reproducibly seen, for example, as in. Figure 4.1.

3.5 Generation of tetracycline inducible 32Dcl3 cells which constitutively express MLL-ENL

In order to study the transcriptional targets of MLL-ENL, it was necessary to generate 32Dcl3 rtTA clones able to inducibly express this fusion protein.

The 9678bp pTRE-MLL-ENL plasmid (pTREMENL) contained the amino acids 27-1444 of human MLL fused to amino acids 1506-2060 of human ENL under control of the TRE, with a c-MYC tag at the N terminus and HA tag at the C terminus as shown in Figure 2.1. As a result of deletion of the first 26 amino acids of MLL, this construct lacked the Menin-binding domain of MLL which was subsequently found to reside within this region. The pTREMENL was created by replacement of the *Sal*1-*Cla*1 fragment of pCSARQ2 with the 441bp *Sal*1-*Cla*1 fragment containing the TRE, and insertion of c-MYC and HA tags. The pTREMENL plasmid was linearised by digestion with the *Sca*1 restriction enzyme.

In order to generate inducible clones expressing MLL-ENL, the 3E 32DTeton clone was co-transfected with the pTet-tTSHygR and pTREMENL. Initially the plasmids were electroporated in molar ratios of 1:15 pTet-tTSHygR and pTREMENL and this 32DMENL-Pop1 grown in hygromycin selection. 32DMENL-Pop1 was then tested in the presence and absence of doxycycline for expression of MLL-ENL. Cells were incubated with doxycycline for 24 hours and Western blot performed using the 9E10 anti-cMYC antibody.

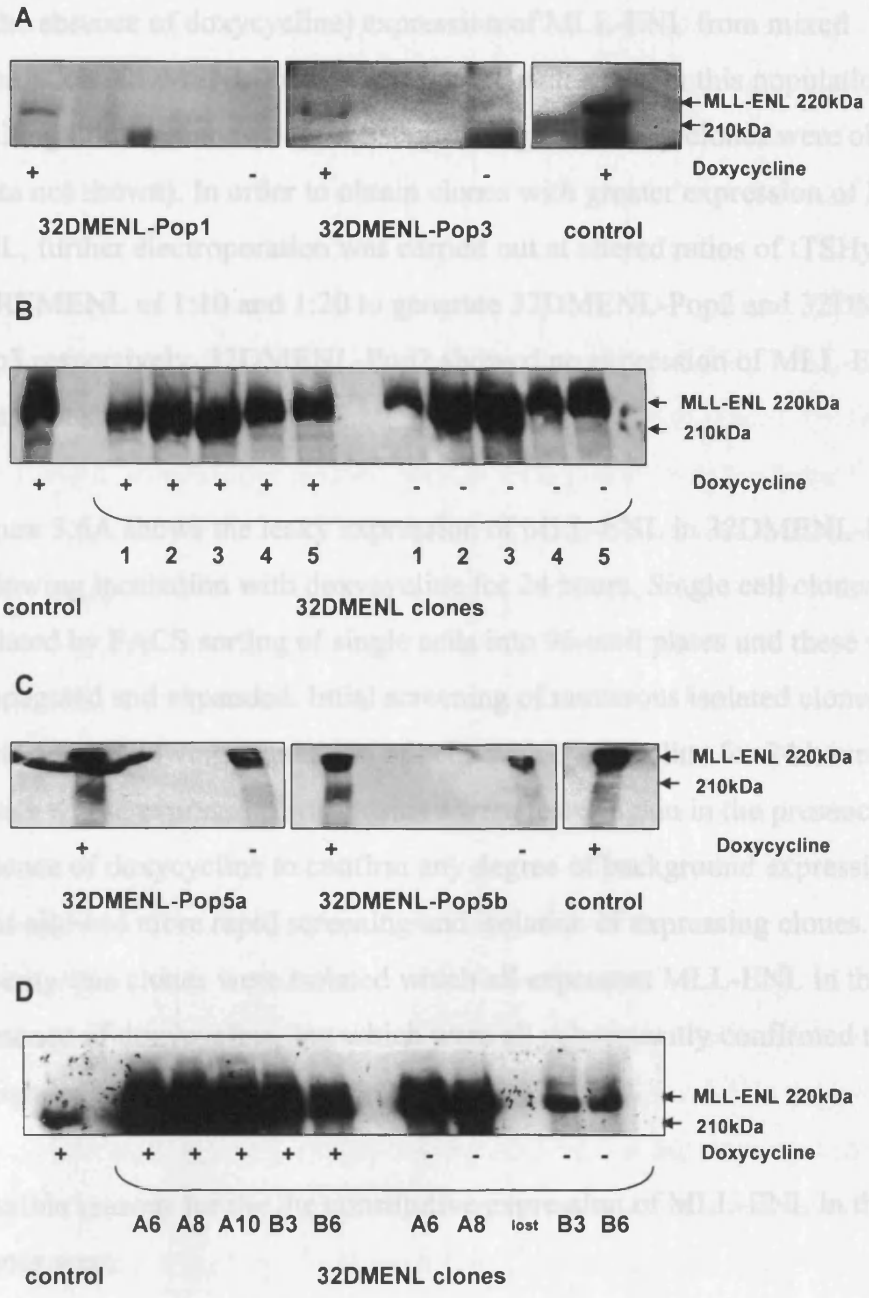


Figure 3.6 Western analysis showing expression of MLL-ENL.

A and **C** show expression of MLL-ENL in initial mixed populations (32DMENL-Pop)

B and **D** show MLL-ENL expression in clones (32DMENL)

Figure 3.6A shows the low level and leaky (showing background expression in the absence of doxycycline) expression of MLL-ENL from mixed population 32DMENL-Pop1. Clones were isolated from this population by limiting dilution and two weakly expressing, non-leaky clones were obtained (data not shown). In order to obtain clones with greater expression of MLL-ENL, further electroporation was carried out at altered ratios of tTSHygR to pTREMENL of 1:10 and 1:20 to generate 32DMENL-Pop2 and 32DMENL-Pop3 respectively. 32DMENL-Pop2 showed no expression of MLL-ENL (data not shown).

Figure 3.6A shows the leaky expression of MLL-ENL in 32DMENL-Pop3 following incubation with doxycycline for 24 hours, Single cell clones were isolated by FACS sorting of single cells into 96-well plates and these were propagated and expanded. Intial screening of numerous isolated clones was performed following incubation of cells with doxycycline for 24 hours. Those whose expression was positive were tested again in the presence and absence of doxycycline to confirm any degree of background expression. This allowed more rapid screening and isolation of expressing clones. Twenty-one clones were isolated which all expressed MLL-ENL in the presence of doxycycline, but which were all subsequently confirmed to have a high level of constitutive expression.

Possible reasons for the the constitutive expression of MLL-ENL in these clones were:

- possible low levels of tetracycline derivatives in the fetal calf serum used for cell culture which may be causing transactivation of MLL-ENL
- the preferential integration of MLL-ENL into transcriptionally active regions of euchromatin

In order to investigate the first possibility, the clones were washed and incubated in Tetracycline-free serum and re-tested for inducible expression of MLL-ENL. However, all these clones continued to have high levels of constitutive expression and MLL-ENL expression in five of these clones is shown in Figure 3.6B.

Further electroporation was carried out in ratios of tTSHygR to pTREMENL of 1:5 and 1:15 to generate 32DMENL-Pop4 and 32DMENL-Pop5 respectively in an effort to again obtain inducible clones. These last two populations were further divided into six mini populations each and tested for inducible expression of MLL-ENL. This was done in order to increase the representation of different clones with a range of genomic integrations that would result in variable levels of inducibility. Both 32DMENL-Pop5a and 32DMENL-Pop5b mini populations showed high level leaky expression as shown in Figure 3.6C. Clones were isolated from 32DMENL-Pop5b by FACS sorting of single cells. However, Figure 3.6D shows the Western analysis in which these 32DMENL clones are seen to have similar levels of constitutive expression to that of clones previously generated.

The experiment was therefore modified to make use of the 32DMENL constitutive clones generated from 32DMENL-Pop5b. mRNA was isolated from eight constitutively expressing clones for gene expression analysis and pooled following cDNA synthesis. Comparison was made to a mixed population generated by electroporation of the same rtTA clone with pTet-tTSHygR only (32DtTS).

The disadvantages of this system compared to a comparison of inducibly expressing clones were that:

- clonal variation would need to be considered and could not be eliminated, although comparison to a mixed population of control cells would limit some error.

- it would not be possible to study the immediate targets of transcription following induction of expression by doxycycline, or the result of different levels of expression at different time points, in order to compare the degree to which a candidate target was up- or down-regulated. The identification of significantly highly changed targets following constitutive expression may, however, prove equally useful, as these candidate targets would subsequently be analysed more rigorously for their role in the leukaemic phenotype.

Such a system of constitutive expression did allow the following advantages:

- the effect of doxycycline itself on changes in gene expression was no longer a confounding variable in the microarray and Q-PCR experiments.
- the effect of doxycycline itself on cell cycle was no longer a difficult variable to control in the study of the effect of MLL-ENL on apoptosis following IL-3 withdrawal, and on 32Dcl3 cell differentiation, and other possible aspects of phenotype.

Figure 3.7 shows eight chosen clones with variable levels of protein expression, and their levels of relative levels of *MLL-ENL* mRNA transcripts analysed by Q-PCR. The fold difference between *MLL-ENL* and control gene (*Gapdh*) is seen to be large for all clones. The levels of *MLL-ENL* mRNA do not appear to correlate with protein expression for each clone, and this may be a result of post-translational modification of gene expression. Total RNA was isolated from each clone in the absence of doxycycline and used for microarray analysis. The detailed outcome of this microarray analysis will be discussed in Chapter 4.

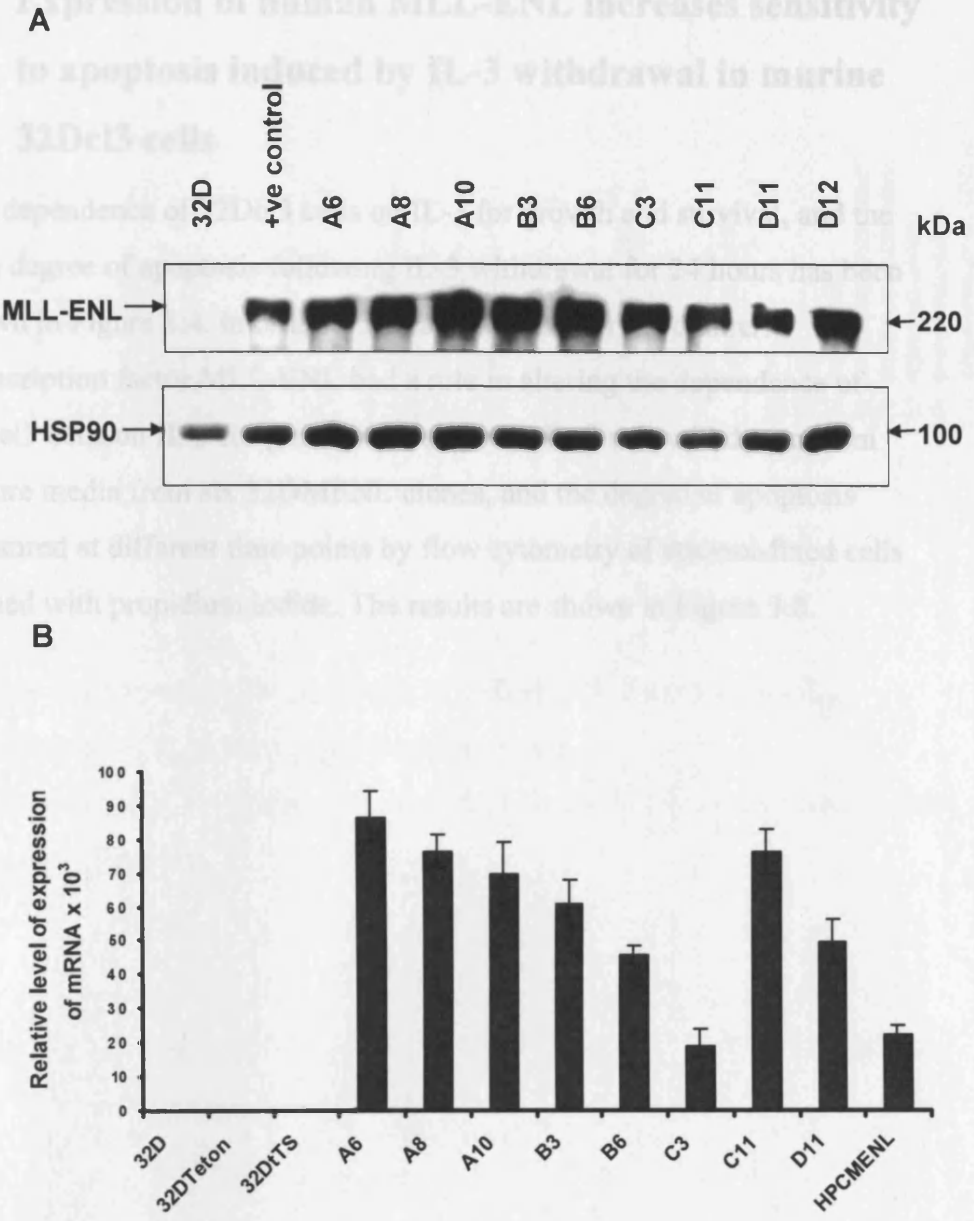


Figure 3.7 32DMENL clones constitutively express MLL-ENL.

A shows the expression of MLL-ENL protein in nine 32DMENL clones labeled as shown

B shows the relative levels of expression of mRNA in each clone by Q-PCR. The figure also shows the level of expression in a positive control (HPCMENL)

3.6 Expression of human MLL-ENL increases sensitivity to apoptosis induced by IL-3 withdrawal in murine 32Dcl3 cells

The dependence of 32Dcl3 cells on IL-3 for growth and survival, and the high degree of apoptosis following IL-3 withdrawal for 24 hours has been shown in Figure 3.4. In order to investigate whether the chimeric transcription factor MLL-ENL had a role in altering the dependence of 32Dcl3 cells on IL-3 for growth and survival, IL-3 was withdrawn from culture media from six 32DMENL clones, and the degree of apoptosis measured at different time points by flow cytometry of ethanol-fixed cells stained with propidium iodide. The results are shown in Figure 3.8.

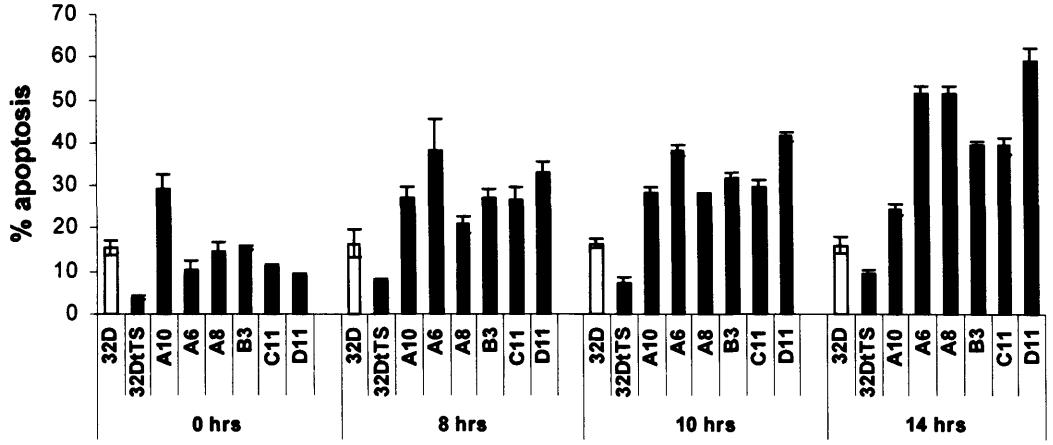


Figure 3.8 Sensitivity to apoptosis of 32DMENL clones following withdrawal of IL-3 from cell culture medium.

($p<0.01$ for all 32DMENL compared to control at 8, 10 and 14 hours, except for A6 at 8 hours).

Compared to the control 32D and 32DtTS cells, the 32DMENL clones showed:

- a significantly increased sensitivity to apoptosis following IL-3 withdrawal, $p<0.01$ at 8, 10 and 14 hours for all clones except A6 at 8 hours.
- an earlier sensitivity to apoptosis after IL-3 withdrawal, which in most clones increased over the time course from 8 hours to 14 hours. All clones except A10 showed significantly increased sensitivity to apoptosis at 8 hours compared to 0 hours ($p<0.01$), A8, B3 and D11 significantly sensitive at 10 hours compared to 8 hours, and all clones were significantly sensitive at 14 hours compared to 10 hours ($p<0.01$). There was no such change in apoptotic sensitivity over the same time course in the 32D and 32DtTS cells.

There was a variation among the 32DMENL clones in regard to their sensitivity to apoptosis which may have been due to their levels of expression of MLL-ENL. However, Western analysis showed no correlation of protein expression levels with apoptotic sensitivity for individual clones.

MLL-ENL thus appears to confer a phenotype on 32Dcl3 cells of increased sensitivity to apoptosis following growth factor withdrawal.

3.7 Expression of human MLL-ENL confers a macrophage-like phenotype in murine 32Dcl3 cells

Arrest of cell differentiation has been shown to be a mechanism involved in leukaemogenesis by a number of oncogenes. A role for MLL-ENL in impeding differentiation has been described in a study of the effect of retroviral MLL-ENL expression in primary mouse haematopoietic cells (Schreiner, *et al* 1999). The addition of G-CSF reversed the maturation block set by MLL-ENL and induced the development of mature granulocytes and

macrophages accompanied by growth arrest. This study also described the need for a further event, in this case cMYC over-expression, before differentiation arrest of the myelomonocytic precursor population was seen. 32Dcl3 cells expressing MLL-AF9 showed an altered *Hox* gene profile following down-regulation of the fusion protein but no morphology was described (Joh, *et al* 1999). Cell lines derived from blasts bearing MLL-AF9 have also been studied for their capacity to differentiate following down regulation of the fusion protein. THP-1 cells did not terminally differentiate following knock down of MLL-AF9 protein (Pession, *et al* 2003), and this may be due to the harbouring of many further acquired mutations in such cells which may make them independent of MLL-AF9 in regard to their malignant behaviour.

The morphology of wild type 32Dcl3 cells and their ability to differentiate into neutrophils in the presence of G-CSF has been shown in Figure 3.3. In order to investigate the effects of MLL-ENL on the differentiation of 32Dcl3 cells, the morphology of Giemsa-stained cytospin preparations prior to and following incubation with G-CSF was studied and compared to that of wild-type 32Dcl3 cells. The results are shown in Figure 3.9. The morphology of 32DMENL clones at day 0 show two appearances; five of eight clones showed increased cytoplasmic vacuolation and cytoplasmic/nuclear ratio. Such macrophage-like features persisted and were accentuated following 6 days of incubation with G-CSF, while features typical of neutrophil differentiation were also seen. Three 32DMENL clones had an appearance similar to the myeloblast-like morphology of parental cells prior to G-CSF incubation and showed neutrophil differentiation at day 6 similar to parental cells.

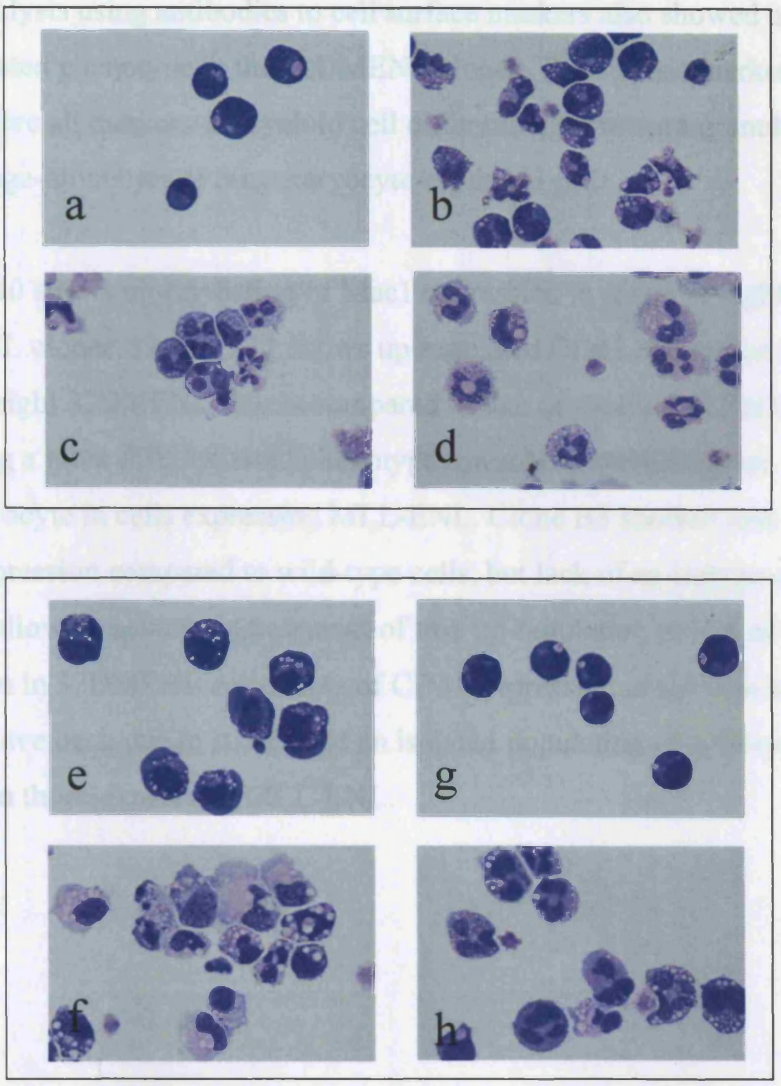


Figure 3.9 Morphology of 32DMENL clones.

Morphology of Giemsa-stained cytospin preparations following incubation with G-CSF shows wild-type 32D cells (a), and subsequent neutrophil differentiation at day 6 (b), day 8 (c), and day 12 (d). Morphology of 32DMENL clones at day 0 shows two appearances; five of eight clones showed increased cytoplasmic vacuolation and cytoplasmic/nuclear ratio (e). At day 6, features typical of neutrophil differentiation, as well as associated gross cytoplasmic vacuolation can be seen (f) The remaining three MLL-ENL clones show myeloblast-like morphology (g) and neutrophil differentiation at day 6 (h) similar to parental cells.

FACS analysis using antibodies to cell surface markers also showed a more differentiated phenotype in the 32DMENL clones. The surface markers studied were all markers of myeloid cell differentiation toward granulocyte, macrophage-monocyte or megakaryocyte-erythroid cells.

Figure 3.10 shows up-regulation of Mac1 expression in seven of eight 32DMENL clones. Figure 3.11 shows up-regulated CD41 expression in seven of eight 32DMENL clones compared to that of parental 32Dcl3 cells, suggesting a more differentiated phenotype towards a macrophage or megakaryocyte in cells expressing MLL-ENL. Clone B3 showed loss of CD41 expression compared to wild-type cells, but lack of an isotype control does not allow an accurate assessment of true up-regulation or loss of CD41 expression in 32DMENL cells. Loss of CD41 expression in the case of clone B3 may have been due to staining of an isolated population of wild-type cells rather than those expressing MLL-ENL.

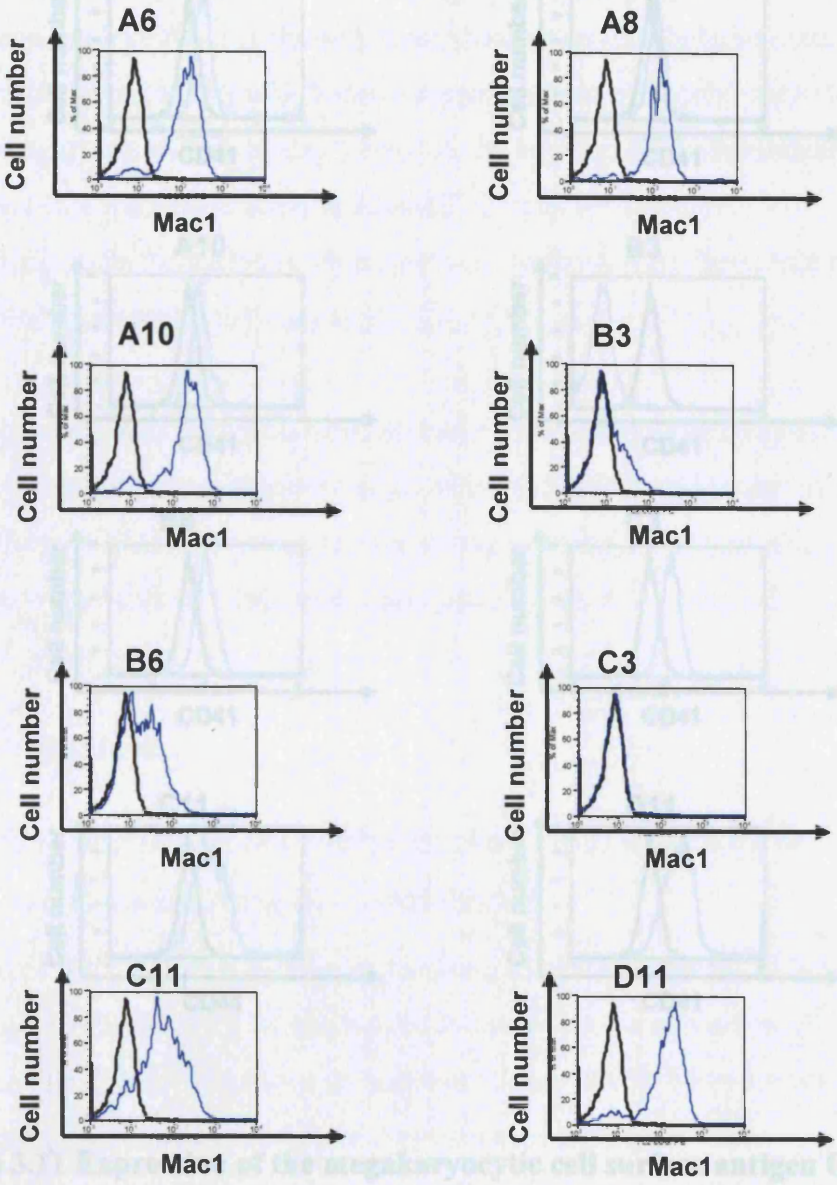


Figure 3.10 Expression of the macrophage cell surface antigen Mac1 in 32DMENL clones.

The expression of Mac1 is shown in eight 32DMENL clones at T=0 (black line: 32Dcl3; blue line: sample, note absence of isotype control).

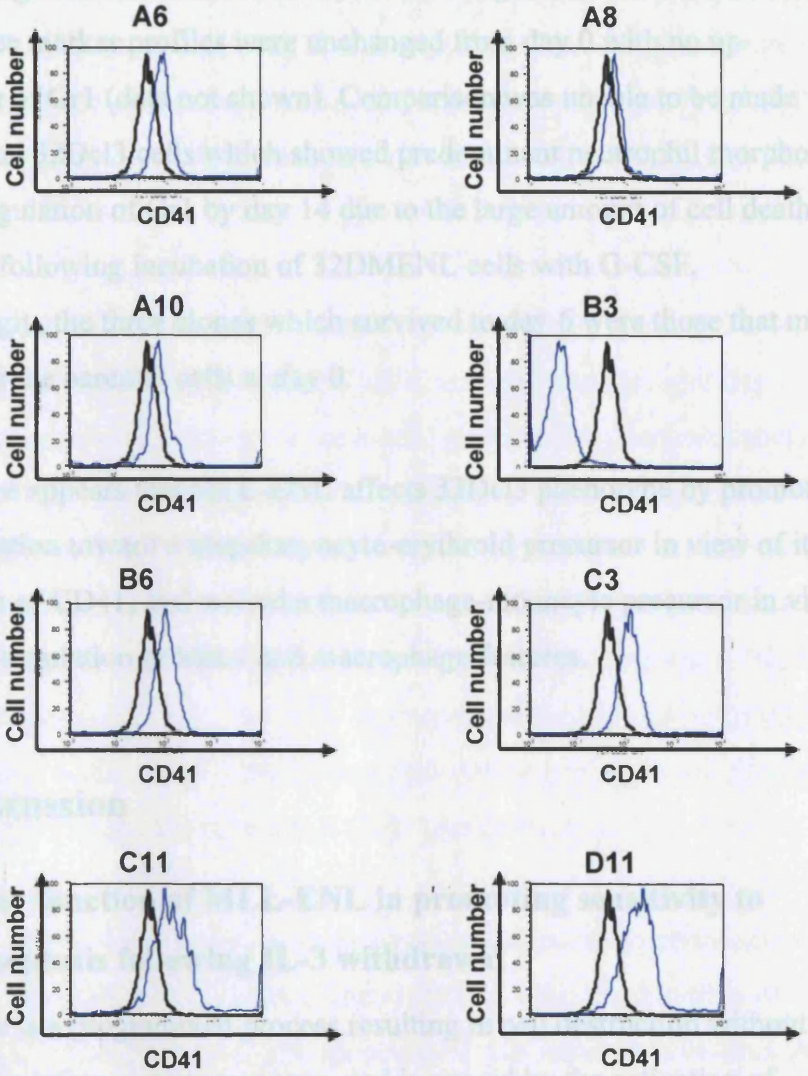


Figure 3.11 Expression of the megakaryocytic cell surface antigen CD41 in 32DMENL clones.

The expression of CD41 is shown in eight 32DMENL clones at T=0 (black line: 32Dcl3; blue line: sample, note absence of isotype control).

Three of eight clones survived to day 6 following treatment with G-CSF, and cell surface marker profiles were unchanged from day 0 with no up-regulation of Gr1 (data not shown). Comparison was unable to be made with the parental 32Dcl3 cells which showed predominant neutrophil morphology and up-regulation of Gr1 by day 14 due to the large amount of cell death observed following incubation of 32DMENL cells with G-CSF. Interestingly, the three clones which survived to day 6 were those that most resembled the parental cells at day 0.

It therefore appears that MLL-ENL affects 32Dcl3 phenotype by promoting differentiation toward a megakaryocyte-erythroid precursor in view of its up-regulation of CD41, and toward a macrophage-monocyte precursor in view of the up-regulation of Mac1 and macrophage features.

3.8 Discussion

3.8.1 The function of MLL-ENL in promoting sensitivity to apoptosis following IL-3 withdrawal

Apoptosis is a programmed process resulting in cell destruction without eliciting an inflammatory response, and is caused by the activation of intracellular cysteine proteases or caspases (Brady 2003). Morphological features of apoptosis include plasma membrane blebbing, volume contraction, nuclear condensation and endonucleolytic cleavage of DNA (Kerr 2002, Kerr, *et al* 1972). Such a process is required for the development and maintenance of normal tissue homeostasis and is tightly interlinked with other essential pathways (Danial and Korsmeyer 2004). The process of normal cell differentiation into specific lineages, as seen in the haematopoietic system, requires the control of death as well as cell division. Deregulation of these processes in a HSC or restricted progenitor to block apoptosis or senescence, or interference with cell cycle to cause hyper-

proliferation may contribute to the leukaemic phenotype. Major apoptotic pathways in haematopoietic cells that are implicated in oncogenesis are the receptor-mediated and mitochondrial-mediated pathways (Brady 2003, Danial and Korsmeyer 2004, Evan and Vousden 2001, Marsden and Strasser 2003).

IL-3 is a cytokine growth factor produced by activated T cells, monocytes/macrophages and stromal cells, and importantly regulates haematopoiesis through its influence on the proliferation, differentiation and survival of various haematopoietic cell lineages and early progenitors (de Groot, *et al* 1998). 32Dcl3 cells were shown to be strictly dependent on IL-3 for growth and survival, and in medium lacking IL-3 these cells undergo apoptosis. IL-3 mediated survival of haematopoietic progenitors or 32Dcl3 cells in vitro appears to be due to the up-regulation of anti-apoptotic proteins BCL2, BCL-X and cMYC, and down-regulation of pro-apoptotic proteins such as BAD (Leverrier, *et al* 1997, Low, *et al* 2001) .

32DMENL cells were shown to have a greater sensitivity to apoptosis following IL-3 withdrawal from culture medium, and de-regulation of apoptosis by MLL-ENL may provide a context for leukaemia to arise. A number of *in vitro* and *in vivo* findings from studies of leukaemia with MLL gene rearrangements point to the importance of the apoptotic process in these leukaemias. Maternal exposure to epipodophylotoxins is associated with infant *MLL*-rearranged leukaemia, while therapy-related short-latency leukaemias and infant leukaemias share a similar clustering of *MLL* genomic breakpoints. The proximity of these sites to topoisomerase II consensus binding sites has implicated topoisomerase II inhibitors directly in the development of *MLL* rearrangements and subsequent leukaemia (Broeker, *et al* 1996). The analysis of *MLL* breakpoints of five t(9,11) non therapy-related childhood AML cases revealed almost identical sequences that were very close to an area susceptible to breakage by topoisomerase II inhibitors,

suggesting that the development of t(9;11) AML involves damage-repair mechanisms (Langer, *et al* 2003). Other studies show that the *MLL* breakpoints seen in therapy-related leukaemias cluster in the same region that is targeted by nucleases of the effector apoptotic pathway. These nucleases are recruited following apoptotic triggers *in vitro* and may be responsible for MLL gene breakage and subsequent formation of fusion genes early in leukaemogenesis without direct involvement of topoisomerase II inhibitors (Betti, *et al* 2001). Cells stimulated to undergo apoptosis following stimulation by an anti-FAS antibody resulted in the formation of the *Mll-Af9* fusion gene following chromatin cleavage at 11q23 at an early stage and evasion of apoptotic execution (Vaughan, *et al* 2005). These mechanisms are not necessarily mutually exclusive and both may contribute to the development these leukaemias.

While a pro-survival effect of over-expressed oncogenes has often been described, a number of oncogenes are pro-apoptotic, this being tissue specific and dependent on the presence of other genetic aberrations. Over-expression of the *cMYC* oncogene has been described as conferring a proliferative advantage leading to cancer in hepatocytes, skin and haematopoietic cells. Subsequent down-regulation of cMYC leads to differentiation or apoptosis, but also to a dormant population which are then allowed to acquire further mutations which confer a definitive survival advantage that is not dependent on the initiating oncogene (Jonkers and Berns 2004). In addition cMYC over-expression in pancreatic islet cells results in apoptosis which can only be rescued by *BCL-X_L*, an anti-apoptotic gene. Yet regression of the tumours that result are dependent on down regulation of cMYC alone (Pelengaris, *et al* 2002). Thus, potentially oncogenic proliferative signals are coupled to a variety of growth-inhibitory processes such as the induction of apoptosis, differentiation or senescence, each of which resists subsequent clonal expansion and neoplastic tumour evolution. Tumour progression occurs only

in the very rare instances where these growth-inhibitory mechanisms are thwarted by compensatory mutations (Evan and Vousden 2001).

Previous studies of the effect of MLL fusion proteins in blasts harbouring MLL-AF9 have shown apoptosis following targeted inhibition of this fusion protein (Kawagoe, *et al* 2001, Pession, *et al* 2003). Targeted inhibition of MLL-ENL in a leukaemic cell line also induced apoptosis (Akao, *et al* 1998) while MLL-AF4 bearing leukaemic cells show resistance to serum-deprivation-induced or interferon gamma regulated FAS-mediated apoptosis. GADD34 expression correlates with, and enhances, apoptosis after treatment with ionizing radiation (Hollander, *et al* 1997), and both MLL-AF9 and MLL-ENL have been shown to abrogate GADD34-induced apoptosis while wild-type MLL causes increased apoptosis (Adler, *et al* 1999). In addition to the abrogation of GADD34-mediated apoptosis, MLL fusion proteins have been shown to abrogate p53-mediated apoptosis also (Wiederschain, *et al* 2005).

The prosurvival effect seen in studies involving leukaemic cell lines derived from patients with MLL translocations may be complicated by the numerous other acquired mutations likely to be present which may themselves be responsible for the survival advantage of the cell rather than it being a direct effect of the MLL fusion. In this study, MLL-ENL is expressed alone without the confounding effects of possible other accumulated mutations, and a pro-apoptotic effect is observed in the context of IL-3 withdrawal. The role of the fusion protein in promoting apoptosis may be to confer a selective pressure for the cell to acquire a mutation in order to survive, and if a cell is able to escape apoptosis by acquiring a survival advantage, it may then go on to cause leukaemia.

The pro-apoptotic effect of isolated expression of an MLL fusion protein is supported by the finding that graded increased expression of MLL-AF4 in

U937 cells results in G1 cell cycle arrest, prolonged doubling time as well as up-regulation of Mac1, thus possibly protecting the cell from etoposide-mediated cytotoxicity (Caslini, *et al* 2004). Studies have also examined the effect of MLL-ENL expression on apoptosis and cell survival in other contexts of apoptotic stimuli causing DNA damage that result in receptor–mediated apoptosis. MLL-ENL function has been shown in murine progenitor cells to substantially increase the incidence of chromosomal abnormalities in proliferating cells that survive exposure to etoposide, also known to be associated with secondary MLL fusion leukaemias. This phenotype is also associated with an altered pattern of cell cycle arrest and/or apoptosis and the authors suggest that MLL fusion proteins render cells more vulnerable to further DNA damage and mutation in the presence of chronic exposure to the agent that induced the MLL fusion itself (Eguchi, *et al* 2006).

Thus apoptosis is a context for further oncogenic mutations that synergise with the fusion protein to form a leukaemic clone, and this may be a mechanism by which MLL-ENL is involved in leukaemogenesis.

3.8.2 The function of MLL-ENL in promoting differentiation towards a macrophage phenotype in 32Dcl3 cells

During G-CSF induced differentiation of 32Dcl3 cells, *Hoxa9*, *Hoxb8*, *Meis1* and *Meis2* were shown to be down-regulated, while enforced expression of *Hox* or *Meis* genes inhibited such differentiation (Fujino, *et al* 2001). Disruption of ubiquitin-mediated degradation of *Hoxa9* and subsequent altered expression levels have also been shown to inhibit G-CSF mediated neutrophil differentiation of 32Dcl3 cells (Zhang, *et al* 2003). Aberrant cellular differentiation may therefore result from the expression of MLL fusion proteins in which normal expression of the MLL gene is lost and such abnormal differentiation may be a result of aberrant *Hox* gene expression. G-CSF induced differentiation of 32Dcl3 cells inducibly expressing MLL-AF9

was shown to result in aberrant *Hox* gene expression with up-regulation of *Hoxa7*, *Hoxb7* and *Hoxc9* genes, but characterisation of the phenotype of the cells was not described in this study (Joh, *et al* 1999). Expression of MLL-AF9 in a leukaemic cell line was associated with maintained levels of *Hoxa7* and *Hoxa10*, and down regulation of these genes followed down-regulation of the fusion protein with resultant apoptosis (Kawagoe, *et al* 2001), but there was no evidence of increased differentiation. Similarly, anti-sense mediated down regulation of MLL-AF9 in the same cell line failed to promote terminal macrophage differentiation, suggesting that early expression of MLL-AF9 may serve to maintain the monocytic phenotype but was not responsible for a differentiation block in these cells (Pession, *et al* 2003).

Other studies suggest that MLL fusion proteins actually promote haematopoietic cell differentiation. Tetracycline-inducible expression of MLL-AF9 in monoblastic U937 cells was associated with differentiation of these cells into macrophages, with up-regulated expression of Mac1 and CD14 and eventual cell death, while expression of N-terminal MLL only was associated with partial macrophage differentiation thought to be attributable to the MLL AT hook-containing region (Caslini, *et al* 2000). Increased differentiation of monoblastic U937 cells with up-regulated Mac1 was also observed in the presence of graded increased conditional expression of MLL-AF4 (Caslini, *et al* 2004).

Studies in which primary cells are transformed by MLL fusions, and results of in vivo mouse models are more conclusive that the fusion proteins cause arrested haematopoietic differentiation. Primary cells transduced with MLL-ENL form an IL-3 myelomonocytic precursor population capable of differentiation by G-CSF, but co-operation of MLL-ENL with cMYC induces a block in differentiation of these cells (Schreiner, *et al* 2001). Furthermore, retroviral transplantation models of MLL-ENL and MLL-

GAS7 have shown that these fusion proteins alone transform multipotent haematopoietic progenitors, as well as more committed progenitors, with resulting impaired differentiation and in vivo leukaemia (Cozzio, *et al* 2003, Lavau, *et al* 1997, So, *et al* 2003a, Zeisig, *et al* 2003b).

In summary, studies investigating the association of expressed MLL fusion proteins and differentiation in conditionally expressing cells or leukaemic cell lines do not demonstrate a definitive role for these proteins in causing maturation arrest. However, arrested differentiation of primary haematopoietic cells transduced with MLL fusion proteins suggests that they mediate such an effect in these cells. The phenotype seen in leukaemic cell lines may be due to the presence of additionally sustained mutations which may be responsible for maintaining arrested differentiation rather than it being due to the MLL fusion protein, although the latter may have had a role at an earlier stage in leukaemogenesis in causing differentiation arrest.

In this study, constitutive expression of MLL-ENL promoted macrophage differentiation of 32Dcl3 cells with up-regulation of Mac1 and the megakaryocyte marker CD41, and morphological features in 5 of 8 clones. Such a phenotype suggests a role for MLL-ENL in disrupting pathways of differentiation in MLL-associated leukaemias, and is in keeping with the promotion of macrophage differentiation by MLL-AF9 and MLL-AF4 in U937 cells (Caslini, *et al* 2004, Caslini, *et al* 2000). However, enhanced differentiation is in contrast to a maturation arrest suggested by increased *Hox* gene expression in 32Dcl3 cells expressing MLL-AF9 (Joh, *et al* 1999). This difference may be a result of the different fusion protein studied, or rather the constitutive nature of MLL-ENL expression in the cells studied currently. It may be that MLL-ENL expression at an initial stage in 32Dcl3 cells drives cells toward a maturation arrest, but that continued constitutive expression results in the activation of downstream events in response to this attempt at arrest. It may be these events that are responsible for the mature

phenotype rather than the MLL fusion itself which may then no longer have a role itself in altered differentiation of the cells. This would mirror the situation seen in the U937 cells studied which are have probably sustained further mutations or downstream activation of events by the fusion proteins. Further incubation of these differentiated cells with G-CSF resulted in observed accelerated cell death which would follow terminal differentiation.

The finding that CD41 is up-regulated by MLL-ENL may be related to the identification of *Gata1* as a candidate target of MLL-ENL following microarray analysis (see Chapter 4). GATA1 is a transcription factor that promotes erythroid and megakaryoblastic differentiation of primary haematopoietic cells, and also has a role in myeloid differentiation. Up-regulated *Gata1* may aberrantly promote megakaryocyte differentiation in 32Dcl3 cells. The effect of attempted megakaryocyte differentiation with thrombopoietin, or of erythroid differentiation with erythropoietin in 32DMENL cells compared to control may support the role of MLL-ENL mediated up-regulation of *Gata1* in promoting aberrant differentiation. In addition, a direct association was recently found between MLL and GATA3 indicating an important role of MLL in regulating GATA family transcription factors and in maintaining memory Th2 cell function as part of immune regulation (Yamashita, *et al* 2006). However, subsequent validation of *Gata1* showed this gene to be undetected by Q-PCR in primary cells transduced with MLL-ENL, making its role less interesting than other genes that were regulated in these cells by the fusion protein, and the association of MLL-ENL with erythroblastic or megakaryocytic leukaemia in humans is also not reported. Therefore, these experiments were deferred in favour of others that had begun to suggest insights into the role of MLL-ENL in leukaemogenesis.

4 Identification of potential targets of the transcriptional fusion proteins MLL-ENL and MLL-AF9

4.1 Introduction

The aim of the project was to identify targets of MLL-ENL and MLL-AF9 in order to understand the mechanism of leukaemogenesis arising from expression of these fusion proteins. As a result, novel genes amenable to molecular targeted therapy may be found. MLL-ENL and MLL-AF9 are likely to regulate the expression of a different subset of genes to those regulated by *MLL* or each partner gene alone. In order to examine targets of these fusion proteins, expression clones expressing MLL-ENL and MLL-AF9 were first generated, as well as control expression clones. Gene expression analysis using microarray technology was then used to identify subsets of genes regulated by the fusion proteins.

4.2 Generation of tetracycline 32Dcl3 cells which inducibly express MLL-AF9 and analysis of gene expression

4.2.1 Generation of MLL-AF9 expression clones

The 8047bp pTRE-MLL-AF9 plasmid (pTREMAF9) contained the amino acids 27-1362 of human MLL fused to amino acids 478-568 of human AF9 under control of the TRE, with a cMYC tag at the N terminus and HA tag at the C terminus as shown in Figure 2.1. The pTREMAF9 plasmid was created by replacement of the *Sal*1-*Cla*1 fragment of pCSHRXAF9 with the 441bp *Sal1-Cla*1 fragment containing the TRE, and insertion of cMYC and HA

tags. The pTREMAF9 plasmid was linearised by digestion with the *Sca*1 restriction enzyme.

A hygromycin resistance gene under control of the SV40 promoter was digested from the pTRE2Hyg plasmid by restriction enzyme digestion with *Xho*1, and ligated to the pTet-tTS plasmid at the *Xho*1 site to create pTet-tTsHygR as shown in Figure 2.1. This plasmid was created in order to eliminate any background expression of the fusion protein that may be toxic to the cells, and which may have caused previous reported failures to generate stable cell lines expressing these proteins (Ayton and Cleary 2001).

In order to generate inducible clones expressing MLL-AF9, the 3E and 8D 32DTet-On clones were co-transfected with the pTet-tTSHygR and pTREMAF9. Initially the plasmids were electroporated in molar ratios of 1:10 tTSHygR: pTREMAF9 and the mixed populations kept as one and not divided. The two mixed populations 32DMAF9-Pop1 and 32DMAF9-Pop2 were grown in hygromycin selection. These populations were then tested in the presence and in the absence of 24 hours of doxycycline for expression of MLL-AF9 by Western blot using the anti-c-MYC antibody 9E10.

Both 32DMAF9-Pop1 and 32DMAF9-Pop2 showed expression of MLL-AF9 with a significant degree of background expression in the absence of doxycycline. Figure 4.1A shows expression in 32DMAF9-Pop1 as a representative example. Single cell clones were isolated from these populations by a process of limiting dilution, the clones expanded and a large number screened for expression of MLL-AF9. Only one clone showed expression of MLL-AF9, and did so very weakly (data not shown).

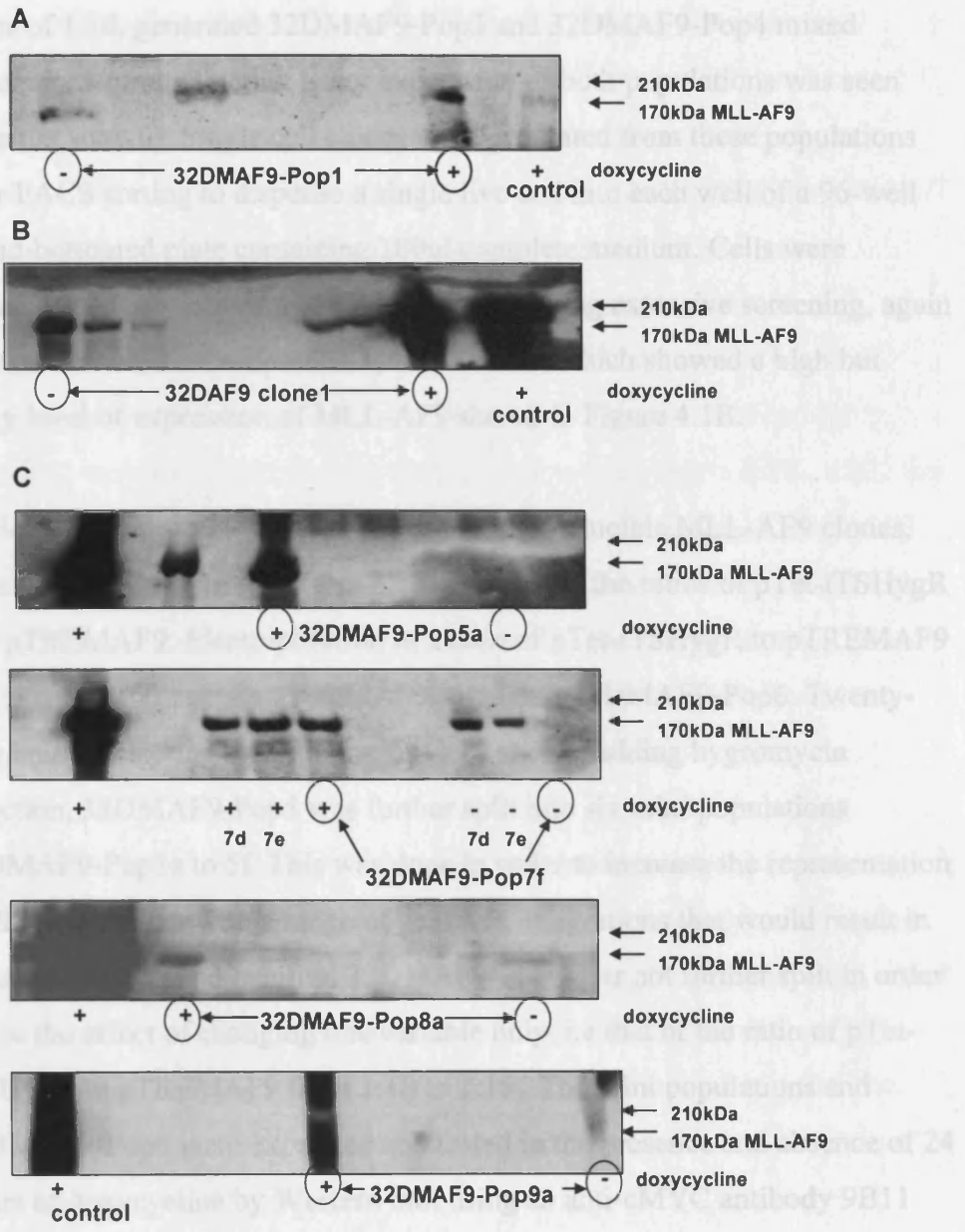


Figure 4.1 Expression of MLL-AF9 in inducible populations.

Western analysis showing

A MLL-AF9 expression in a mixed population 32DMAF9-Pop1;

B MLL-AF9 expression in a 32DMAF9 clone;

C MLL-AF9 expression in 32DMAF9 mini populations.

Further electroporations of pTet-tTSHygR and pTREMAF9, again in molar ratios of 1:10, generated 32DMAF9-Pop3 and 32DMAF9-Pop4 mixed populations. Inducible but leaky expression of both populations was seen (data not shown). Single cell clones were generated from these populations after FACS sorting to dispense a single live cell into each well of a 96-well round-bottomed plate containing 100ul complete medium. Cells were subsequently propagated and expanded. Following extensive screening, again only one clone, 32MAF9 clone 1, was isolated which showed a high but leaky level of expression of MLL-AF9 shown in Figure 4.1B.

In view of the difficulty thus far in generating inducible MLL-AF9 clones, the electroporation strategy was modified to vary the ratios of pTet-tTSHygR and pTREMAF9. Electroporation in a ratio of pTet-tTSHygR to pTREMAF9 of 1:5 and 1:15 generated 32DMAF9-Pop5 and 32DMAF9-Pop6. Twenty-four hours following electroporation, just prior to adding hygromycin selection, 32DMAF9-Pop5 was further split into six mini populations 32DMAF9-Pop5a to 5f. This was done in order to increase the representation of different clones with a range of genomic integrations that would result in variable levels of inducibility. 32DMAF9-Pop6 was not further split in order to see the effect of changing one variable only, i.e that of the ratio of pTet-tTSHygR to pTREMAF9 from 1:10 to 1:15. The mini populations and 32DMAF9-Pop6 were expanded and tested in the presence and absence of 24 hours of doxycycline by Western blot using an anti-cMYC antibody 9B11 that had greater affinity for the cMYC epitope. One mini population, 32DMAF9-Pop5a, showed a high level of induction of MLL-AF9 with no expression in the absence of doxycycline, shown in Figure 4.1C. However, generation of single cell clones by FACS sorting into 96 well plates, expansion, and extensive screening of these did not result in any expression of MLL-AF9.

The isolation of single cell clones expressing MLL-AF9 appeared to be highly problematic so the aim was modified to generate inducible mini populations instead, and to use the isolated mRNA for gene expression analysis. In view of previous generation of a mixed population following electroporation in the ratio of 1:5 tTSHygR to pTREMAF9, 32DMAF9-Pop7 was generated in the same way. This population was split into mini populations 32DMAF9-Pop7a to 7f prior to hygromycin selection and expanded. These were tested in the presence and absence of 24 hours of doxycycline by Western blot. Mini population 7f was found to be highly inducible with no background expression as shown in Figure 4.1C, while the remaining five mini populations all showed leaky expression of MLL-AF9. Equal loading of protein was demonstrated by Ponceau staining of the membrane and confirmed for all populations tested (data not shown). The mRNA isolated from 32DMAF9-Pop7f in the presence and absence of doxycycline was used for microarray analysis.

Following a further electroporation two additional mini populations, 32DMAF9-Pop8a and 2DMAF9-Pop9a, were generated in the same way. Figure 4.1C shows that 8a had a low level background expression with a high level of induction, while 9a showed a low level of induction with no background expression. mRNA was isolated from both these populations also in the presence and absence of doxycycline and used for microarray analysis.

4.2.2 Expression of MLL-AF9 protein in murine 32Dcl3 cells is lost following maintenance of cells in culture

There appeared to be a problem in maintaining expression of the MLL-AF9 protein. Some of the mixed and mini populations initially expressed the protein and then failed to do so after a period of time in culture. Examples included 32DMAF9-Pop5a which was maintained in culture for ten days after which the initially high expression was not at all detectable and all

single cell clones were negative (data not shown). This population showed leaky expression when a lysate frozen at the time of initial expression was thawed and tested (data not shown). Mini populations 7d and 7e were two of five strongly expressing and leaky populations that subsequently expressed much more weakly, and did not express at all on further testing as seen in Figure 4.2A. This was very problematic as regards generation of inducible single cell clones for microarray analysis or functional studies.

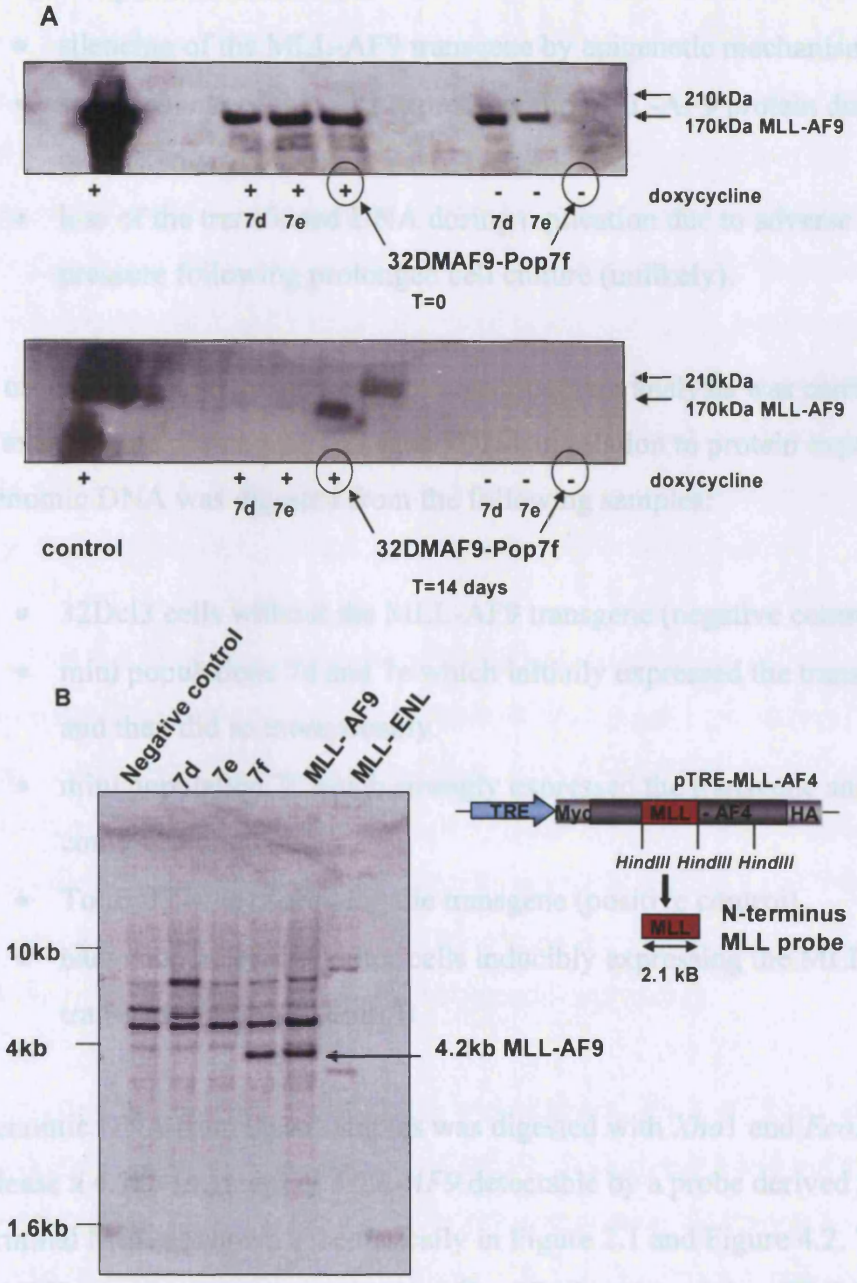


Figure 4.2 Investigation of lost expression in MLL-AF9 populations.
A shows a Western analysis of MLL-AF9 expression in 32DMAF9 mini-populations at initial testing, and after 14 days in culture.
B shows Southern analysis to determine the presence of *MLL-AF9* transgene at the time of absent protein expression.

Possible explanations included:

- silencing of the MLL-AF9 transgene by epigenetic mechanisms
- selective loss of the cells expressing the MLL-AF9 protein due to cytotoxicity to the cell
- loss of the transfected DNA during replication due to adverse selective pressure following prolonged cell culture (unlikely).

In order to investigate these possibilities, Southern analysis was carried out to examine the presence of transgene DNA in relation to protein expression. Genomic DNA was digested from the following samples:

- 32Dcl3 cells without the MLL-AF9 transgene (negative control)
- mini populations 7d and 7e which initially expressed the transgene and then did so more weakly.
- mini population 7f which strongly expressed the transgene and still continued to do so
- TonBaf3 cells expressing the transgene (positive control)
- haematopoietic progenitor cells inducibly expressing the MLL-ENL transgene (positive control)

Genomic DNA from these samples was digested with *Xho*1 and *EcoR*1 to release a 4.2kb fragment of *MLL-AF9* detectable by a probe derived from N-terminal MLL as shown schematically in Figure 2.1 and Figure 4.2. The hybridisation probe was prepared by *Hind*III digestion of the pTRE-MLL-AF4 vector expressing MLL-AF4 under the TRE (Figure 2.1). This released a 2.1kb fragment of MLL which was gel purified and radioactively labelled with ^{32}P and used as a hybridisation probe for the *MLL-AF9* transgene. Southern analysis for the presence of the *MLL-AF9* transgene, together with simultaneous Western analysis for the expression of the protein is shown in Figure 4.2. Following initial generation of the 32DMAF9 populations, high background expression of MLL-AF9 is seen in 32DMAF9-Pop7d and 7e,

and inducible expression seen by 7f. A repeat Western after the cells had been for 14 days in culture shows no protein expression of 7d and 7e, while inducible expression of 7f remains (Figure 4.2A). Corresponding Southern analysis shown in Figure 4.2B demonstrates that the presence of the transgene correlates with protein expression, while absence of the transgene correlates with absent protein expression. It therefore appeared that cells containing the MLL-AF9 transgene were being lost due to cytotoxicity to the cell as this seemed to occur in cells showing leaky expression, and the selective pressure of a high concentration of hygromycin may additionally be contributing to such cell death. The cells remained neomycin resistant so it was unlikely that the rtTA transactivator gene was being lost.

In order to further investigate if cells with leaky expression of MLL-AF9 could survive more easily in the absence of the selective pressure of hygromycin, two leaky mini populations 32DMAF9-Pop5e and 5f were grown in the presence and absence of hygromycin. However, Western analysis did not reveal a difference in the expression of MLL-AF9 in the presence or absence of hygromycin by population 5e and 5f (data not shown), which also showed high background expression.

It appears likely that cells with leaky expression of MLL-AF9 are selected against within a mixed population of cells both expressing and not expressing the transgene. This resulted in loss of cells expressing the transgene over a period of time and led to modifications of the microarray experiment as described.

4.2.3 Experimental strategy for MLL-AF9 microarray experiments

The experimental strategy for MLL-AF9 experiments is shown in Figure 4.3. Three 32DMAF9 mixed populations inducibly expressing MLL-AF9 were cultured with doxycycline to induce gene expression, and three reference 32DTTSHyg mixed populations were also cultured in the presence and absence of doxycycline to allow exclusion of targets of doxycycline only. Total RNA was extracted and processed and analysed by microarray in comparison to cells cultured in the absence of doxycycline. The three inducible populations were grouped as replicates so that pair-wise comparisons of the samples treated with and without doxycycline could be made to identify differentially expressed genes.

An overview of microarray technology is shown in Figure 2.2 and samples were prepared according to the method outlined in Chapter 2. An overview of the strategy for MLL-AF9 microarray experiments is shown in Figure 4.3.

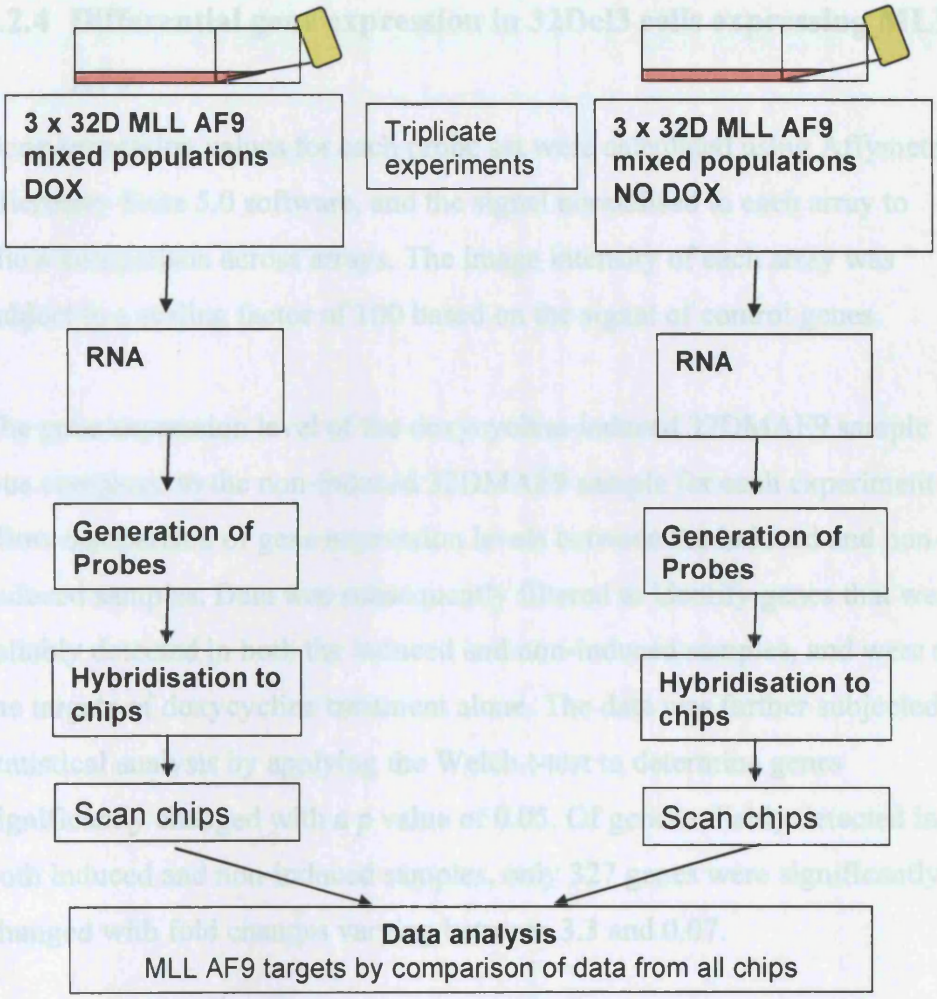


Figure 4.3 Experimental strategy for MLL-AF9 microarray experiments.

4.2.4 Differential gene expression in 32Dcl3 cells expressing MLL-AF9

Gene expression values for each probe set were calculated using Affymetrix Microarry Suite 5.0 software, and the signal normalised to each array to allow comparison across arrays. The image intensity of each array was subject to a scaling factor of 100 based on the signal of control genes.

The gene expression level of the doxycycline-induced 32DMAF9 sample was compared to the non-induced 32DMAF9 sample for each experiment to allow comparison of gene expression levels between the induced and non-induced samples. Data was subsequently filtered to identify genes that were reliably detected in both the induced and non-induced samples, and were not the targets of doxycycline treatment alone. The data was further subjected to statistical analysis by applying the Welch t-test to determine genes significantly changed with a p value of 0.05. Of genes reliably detected in both induced and non-induced samples, only 327 genes were significantly changed with fold changes varying between 3.3 and 0.07.

In order for potentially relevant genes to be detected, the level of significance was relaxed. 665 genes passed the Welch t-test at a significance level where p was less than 0.1, and fold change between induced and non-induced samples ranged from 6.6 to 0.16. From this list, genes were identified as candidate targets according to the following criteria:

- fold change of ≥ 1.4 between induced and non-induced sample
- a potential role in normal or malignant haematopoiesis

4.2.5 Genes up-regulated by MLL-AF9

Of the 665 genes, 8 were identified which were up-regulated and fulfilled the above criteria, and their fold changes varied between 1.4 and 6.7. These

genes are shown in Table 8. 421 genes were up-regulated by ≥1.4 fold but of these all except the 8 genes shown had no known function, or their function did not suggest a potential role in haematopoiesis, and were therefore excluded as potential candidate genes.

Table 8 Genes up-regulated and down-regulated by MLL-AF9

Probe set ID	Fold change	Gene symbol	Notes
1425618_at	6.7	**Dhx9**	RNA helicase, regulation of transcription, DNA-dependent, spermatogenesis
1442604_at	2.7	**Ercc6**	Pyrimidine dimer repair, excision repair cross-complementing rodent repair deficiency, complementation group 6
1446499_at	1.8	**Fadd**	FAS (TNFRSF6)-associated via death domain, regulation of apoptosis, signal transduction
1443792_at	1.8	**Pip5k2b**	phosphatidylinositol metabolism
1419607_at	1.7	**Tnf**	tumor necrosis factor, cell proliferation cytokine and chemokine mediated signaling pathway, induction of apoptosis via death domain receptors, inflammatory response
1426329_s_at	1.6	**Baalc**	brain and acute leukemia, cytoplasmic. Predicts poor survival in AML normal cytogenetics, expressed BM CD34+, downregulated with differentiation

1452844_at	1.4	**Pou6f1**	POU domain, class 6, transcription factor 1, regulation of transcription
1443943_at	1.4	**Plag1**	pleiomorphic adenoma gene 1, *Plag1* and *Plag2* cooperating oncogenes with *CBFbeta-SMMHC* in AML inv (16), mouse model
1455853_at	0.6	**Cdk4**	positive regulation of cell proliferation
1457929_at	0.4	**Mdm2**	cell growth and maintenance, protein catabolism, protein ubiquitination, traversing start control point of mitotic cell cycle
1453928_at	0.2	**Gli3**	brain /CNS development, negative regulation of transcription

4.2.6 Genes down-regulated by MLL-AF9

Of the 665 genes, 3 genes were identified which were down-regulated and fulfilled the above criteria of having a known function of potential relevance to haematopoiesis, and their fold changes varied between 0.2 and 0.6. These genes are shown in Table 8.

4.2.7 Expression of previously identified targets of MLL-AF9

A comparison of genes identified in the current study was made with those highlighted in published studies. The caveats to such comparisons will be discussed in the following section regarding MLL-ENL candidate genes, but no genes in the current study were identified by other studies. In addition, it was noted that there was a very low level of expression of all genes turned on by MLL-AF9 only, as well as those whose expression may have been altered by MLL-AF9 as well as doxycycline, ie both significant and non-significant genes. In addition there were poor degrees of fold change for all the genes. Reasons for this may have included:

- the time point of induction of MLL-AF9 expression was too early at 24 hours. However, Western blotting had shown high levels of protein expression at this time point.
- the quality of material was poor. This was unlikely as the material underwent various quality control tests before hybridisation to the microarrays.
- the 32Dcl3 myeloid progenitor cell did not represent an appropriate stage of myeloid differentiation. No previous studies using this cell line for microarray screening have been perfomed, but MLL-AF9 has been expressed in this cell line and Hox gene analysis carried out by Q-PCR (Joh, *et al* 1999).

While candidate genes have been chosen according to the criteria described, Q-PCR analysis will be required to assess their validity, and further study of

the biological significance of valid candidate genes carried out. In order to allow completion of work into the mechanism of leukaemogenesis by MLL-ENL, further work related to targets of MLL-AF9 will be continued by another member of the group.

4.3 Analysis of gene expression in 32Dcl3 cells expressing MLL-ENL

4.3.1 Experimental strategy for MLL-ENL microarray experiments

The generation of MLL-ENL expression clones has been described in Chapter 3. The experimental strategy for MLL-ENL microarray experiments is shown in Figure 2.2. Eight 32DMENL clones constitutively expressing MLL-ENL, and one reference 32DTtsHyg mixed population were harvested and total RNA extracted. The clones were not incubated with doxycycline as they constitutively expressed MLL-ENL as described in Chapter 3. The cDNAs from each of the eight 32DMENL clones were combined prior to the generation of cRNA which underwent further processing and hybridisation to microarrays, and compared to that of the reference mixed population. The experiment was performed in triplicate, and the three experimental samples grouped as replicates to allow comparison with the control samples. As gene expression could not be analysed following abolition of MLL-ENL expression, this method would not allow discrimination of primary or secondary targets of the fusion protein. However, candidate targets were subsequently analysed in HPC where MLL-ENL expression was inducible and tightly controlled.

Samples were prepared according to the method outlined in Chapter 2 and an overview of the experimental strategy for MLL-ENL experiments is shown in Figure 4.4.

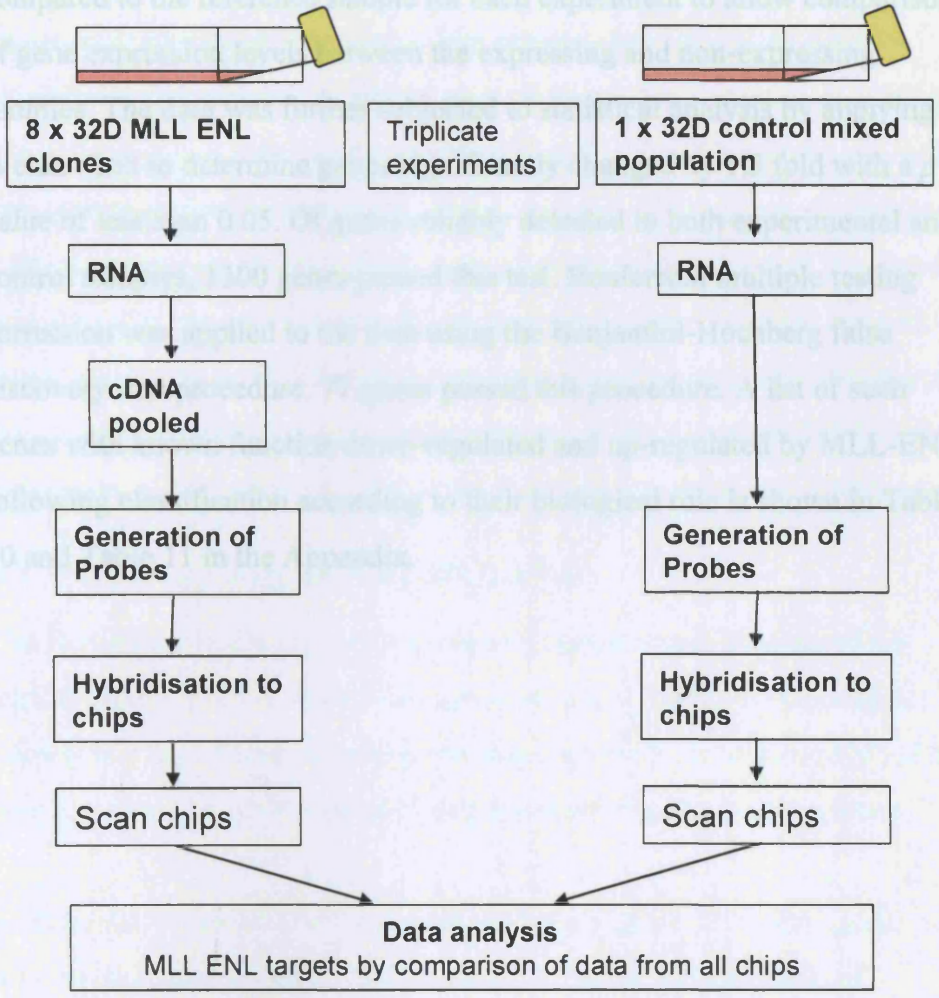


Figure 4.4 Experimental strategy for MLL-ENL microarray experiments.

The gene expression level of the pooled 32DMENL constitutive sample was compared to the reference sample for each experiment to allow comparison of gene expression levels between the expressing and non-expressing samples. The data was further subjected to statistical analysis by applying the Welch t-test to determine genes significantly changed by 1.5 fold with a p value of less than 0.05. Of genes reliably detected in both experimental and control samples, 1300 genes passed this test. Bonferroni multiple testing correction was applied to the data using the Benjamini-Hochberg false discovery rate procedure. 77 genes passed this procedure. A list of such genes with known function down-regulated and up-regulated by MLL-ENL following classification according to their biological role is shown in Table 10 and Table 11 in the Appendix.

4.3.2 Genes down-regulated by MLL-ENL

Genes were identified according to the criteria above that were down-regulated in the presence of a high level of constitutive expression of MLL-ENL. These genes may be of great interest and biological significance and will require further validation by another member of the group. Validation of genes up-regulated by MLL-ENL were given priority in view of the putative role of fusion proteins to transactivate their target genes, and the opportunity arose to focus on some identified genes that showed highly significant and high levels of expression seen in the presence of high levels of MLL-ENL expression, these genes also appearing to be of high biological significance.

4.3.3 Genes up-regulated by MLL-ENL

The Benjamini-Hochberg test is extremely rigorous, and absence of up-regulation of a gene in one of the eight clones may result in that gene not passing this test. Some of these genes may, however, be biologically valid and significant but may result in being excluded due to clonal variation.

As described, data was first analysed to identify genes that were significantly only up-regulated by MLL-ENL. This would allow identification of candidate target genes whose over-expression may be related to the phenotype observed in 32DMENL cells, and up-regulation of these genes may also be present in primary cells transduced with MLL-ENL. Down-regulation of these candidate targets in 32DMENL cells, and primary cells transduced with MLL-ENL would be strong evidence to support their role as a direct target of MLL-ENL and their potentially significant role in leukaemogenesis that would require further analysis.

Therefore, criteria were used to identify candidate targets from both the list of 77 highly significantly changed genes, as well as from the list of 1300 genes which passed the Welch t-test. These criteria were:

- genes known to have a role in normal or malignant haematopoiesis
- genes up-regulated in the experimental sample by a fold change of greater or equal to 10.

Genes significantly up-regulated by MLL-ENL and chosen as candidate targets according to the above criteria are shown in Table 9. Two of these genes, (*Apo J* and *HTm4*), passed the Benjamini-Hochberg false discovery rate procedure.

Table 9 Candidate target genes up-regulated by MLL-ENL

Probe set ID	*P*-value	Fold change	Gene symbol
1454849_at	0.0004	**154.8**	**ApoJ**
1421441_at	0.0064	**103.5**	**Agpt**
1420572_at	0.0004	**82.9**	**HTm4**
1450297_at	0.0054	**71.7**	**Il-6**
1449232_at	0.0062	**70.5**	**Gata1**
1417714_at	0.0031	**63.8**	**Hba-a1**
1415960_at	0.0028	**18.7**	**Mpo**

4.3.4 Expression of previously identified targets of MLL-ENL

A number of studies have described gene expression profiles of patient samples of ALL, AML and MLL-rearranged leukaemias or leukaemic cell lines harbouring MLL translocations, and one study to date investigates the profile of primary haematopoietic cells transformed by MLL-ENL (Zeisig, *et al* 2004). These studies are discussed in Chapter 1.

These previously published gene expression analyses were reviewed to see if any of the currently identified targets were also duplicated in these studies. This would possibly allow an insight into the role of such candidate targets depending on the context of the experiments in which such genes were found. The context is important to consider as many of these studies analyse human leukaemic blasts at the time of diagnosis, or leukaemic cell lines which have undergone multiple mutations associated with tumour initiation and progression. The cells currently studied are at an early stage of differentiation and express the MLL fusion gene alone with no other secondary mutations, and retain IL-3 dependence suggesting that they might be at an early stage in the leukaemia process. In addition the primary haematopoietic cells transformed by MLL-ENL and analysed by microarray (Zeisig, *et al* 2004) are not strictly comparable to the myeloid precursor cells constitutively expressing full-length MLL-ENL used here.

Only two genes highlighted by the above published studies were found in this current study. These were *Il-6* and *Mpo*, both of which were chosen as candidate genes according to criteria described. *Il-6* was up-regulated when MLL-ENL expression was silenced in a conditional model (Zeisig, *et al* 2004). *Mpo* was up-regulated in MLL-AML patient blasts (Debernardi, *et al* 2003). None of the *Hox* genes highlighted in the above studies were detected in the current study but the caveats of comparisons between this and previous studies have been outlined. A possible reason for the failure to detect Hox

gene changes in the current system using microarrays is that they may be already down-regulated in these 32DMENL cells which partially show macrophage features. The ability to perform an extensive Q-PCR profile of *Hox* genes (in collaboration with the Haematology Department, CCRCB, Queen's University, Belfast) has revealed a low level of expression of a number of *Hox* genes in 32Dcl3 cells (data not shown). Up-regulation of *Hoxa7, Hoxb7* and *Hoxc9* in 32Dcl3 cells incubated with G-CSF has been shown to be inhibited by MLL-AF9 expression (Joh, *et al* 1999), and in this study Q-PCR was used to determine alterations in *Hox* gene expression in HPC expressing MLL-ENL where *Hox* genes could be measured in a control population. These experiments will be discussed in Chapter 5.

4.4 Potential target genes of MLL-ENL are validated by Q-PCR

Real time Q-PCR reliably detects and measures products generated during each cycle of the PCR which are directly proportional to the amount of template prior to the start of the PCR process (Ginzinger 2002). Levels of mRNA of the candidate target genes were measured using cDNA derived by reverse transcription of total RNA from the eight 32DMENL clones which had previously been pooled for microarray experiments. Target signals were compared to those in a 32DtTS reference sample, and so the level to which each target gene was up-regulated in each clone could be measured to validate that the candidate gene was changed.

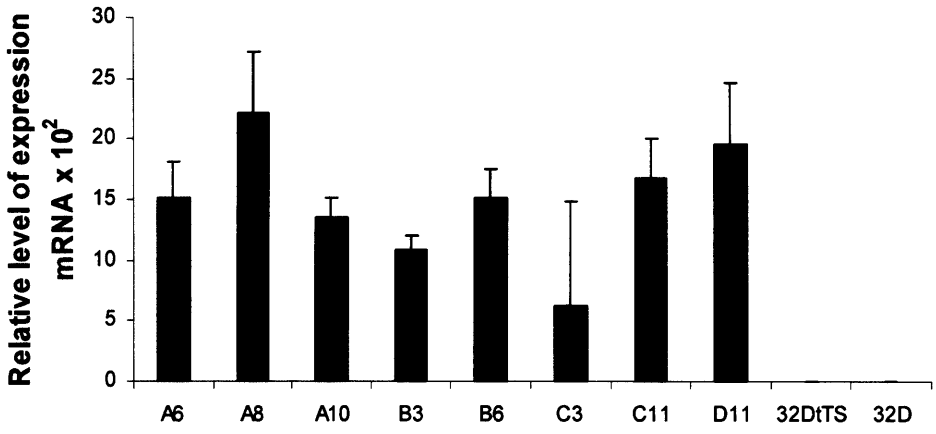
Relative quantitation was carried out to compare the gene of interest with a control gene (*Gapdh*) within each cDNA sample.

All candidate target genes except *Hba1* and *Mpo* were found to be expressed at higher levels in the 32DMENL clones compared to control as shown in Figure 4.5. The fold difference in expression of the candidate genes varied

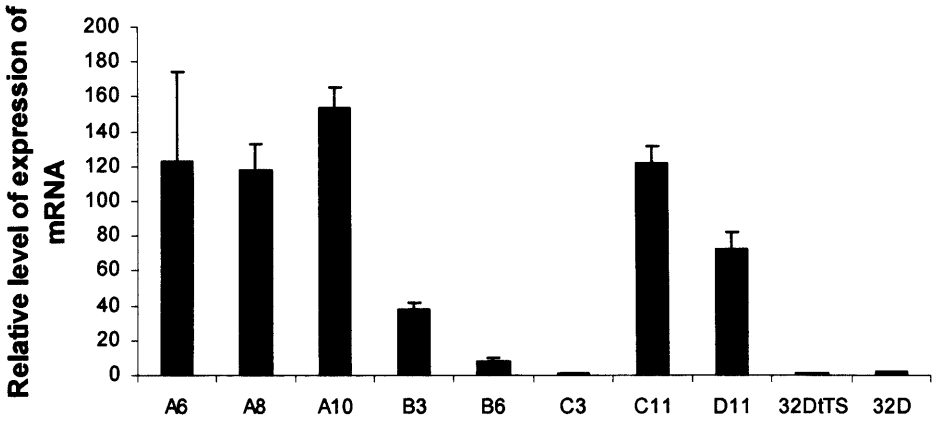
with the gene studied, so that *Gata1* and *Apo J*, were maximally expressed 8-12000 fold compared to control, whereas *Il-6* and *HTm4* were up-regulated 100-120 fold. There also appeared to be variation between clones in regard to expression levels. Fold change of each of the target genes did not appear to correlate with fold changes in MLL-ENL expression measured by Q-PCR for each clone.

Figure 4.5 Q PCR showing relative quantitation of mRNA for each candidate target gene in each 32DMENL clone.

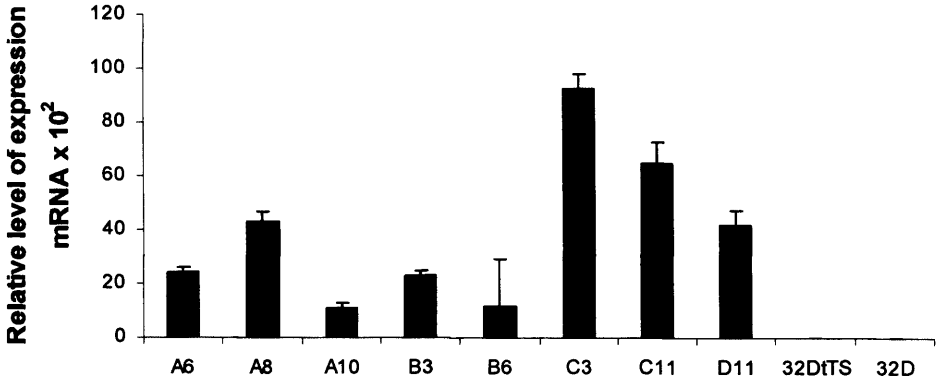
A *Gata1*



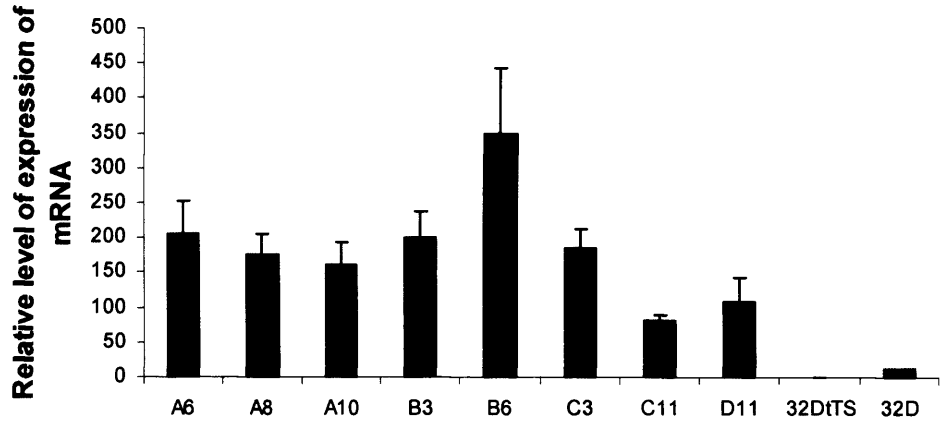
B *HTm4*



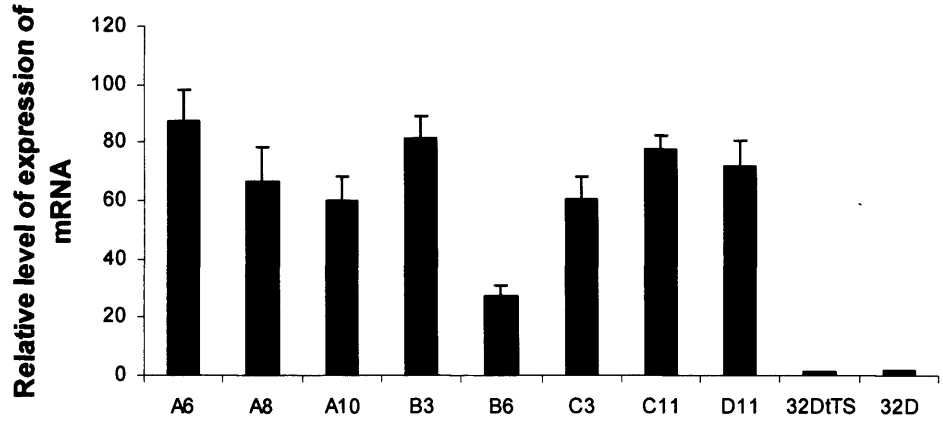
C *ApoJ*



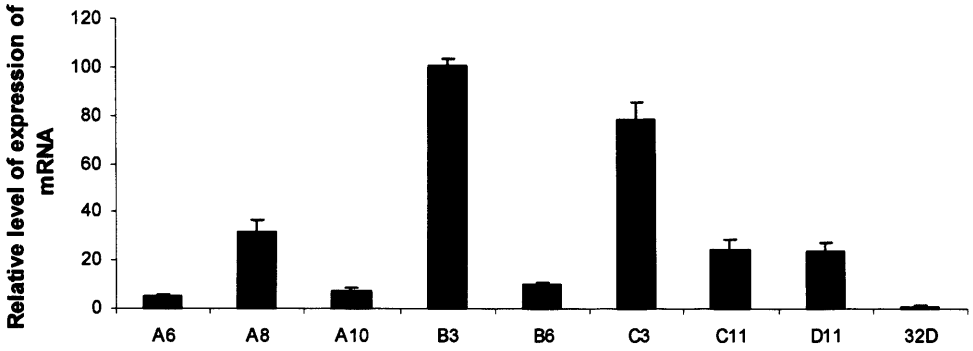
D *Agpt1*



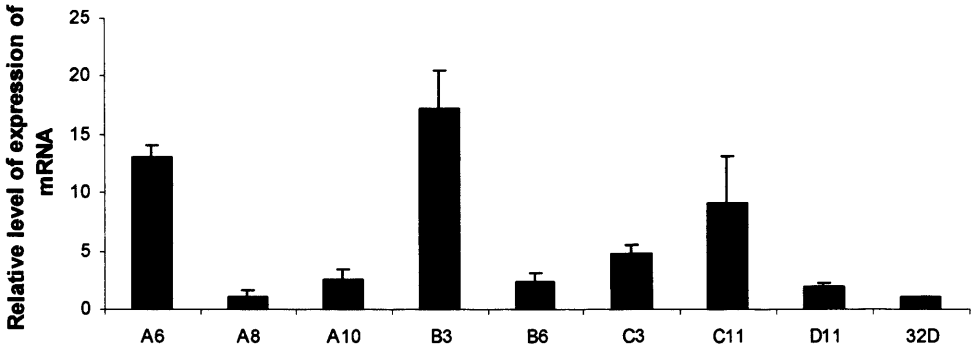
E *Il-6*



F *Hba1*


Relative level of expression of mRNA
120
100
80
60
40
20
0
A6
A8
A10
B3
B6
C3
C11
D11
32D

G *Mpo*


Relative level of expression of mRNA
25
20
15
10
5
0
A6
A8
A10
B3
B6
C3
C11
D11
32D

4.4.1 MLL-ENL is down-regulated following lentiviral delivery of shRNA

Technical difficulties resulting in problems in achieving stable expression of shRNAs against genes of interest prompted the use of a microRNA system whose expression was driven by a polymerase II. A system of pPRIME-SFFV vector delivery of miRNA embedded shRNA was used to down-regulate the expression of MLL-ENL in 32DMENL cells. In addition, to enhance the efficiency of expression of the shRNA transduction and expression, the pRIME vector was modified to express the miRNA from the U3 part of the SFFV strain LTR sequence (SFFV-U3LTR). The GFP was expressed upstream of the WPRE and efficiency of lentiviral infection was assessed by flow cytometric analysis of GFP.

Experiments involving RNA interference of gene expression were carried out in collaboration with Dr. Jasper de Boer.

32DMENL cells were infected with pRIME-SFFV vectors expressing two different miR-shRNAs against human *ENL* (referred to as shENL 1 and shENL 2), miR-shRNA against *MLL* (shMLL), and against *E2A-HLF* control (shHLF). The lentiviral constructs used to down-regulate MLL-ENL by shRNA are shown schematically in Figure 2.3.

The expression of these miRNAs were under the control of the SFFV-U3LTR. The miR-shRNAs were cloned into the lentivirus and the lentiviral construct was co-transfected into a 293T producer cell line with psPAX2 containing the packaging protein and pMD2G containing the envelope protein required for lentiviral function, and which were on separate individual plasmids to reduce the chance of recombination into the retrovirus. The viral supernatant was concentrated by ultrafiltration, and centrifugation-mediated transduction of 32DMENL cells carried out with the addition of

polycationic polybrene to increase transduction efficiency, and recombinant IL-3 to optimize cell growth. Sustained high expression of each miRNA construct was measured by flow cytometry of GFP at days 1, 7 and 14, and cells were sorted by FACS for GFP expression at 14 days. Figure 4.6A shows the levels of stable GFP expression at 14 days of cell culture reflecting stable expression of the miRNA constructs over time. Figure 4.6B shows a high level of GFP expression in FACS sorted cells reflected a high level of infection with the pRIME-SFFV vectors. Thus, in the absence of an inducible system of expression of the MLL-fusion genes, RNA interference was a way in which expression of specific genes could be down-regulated and downstream targets of the MLL fusions examined in a similar way to inhibiting expression in an inducible system.

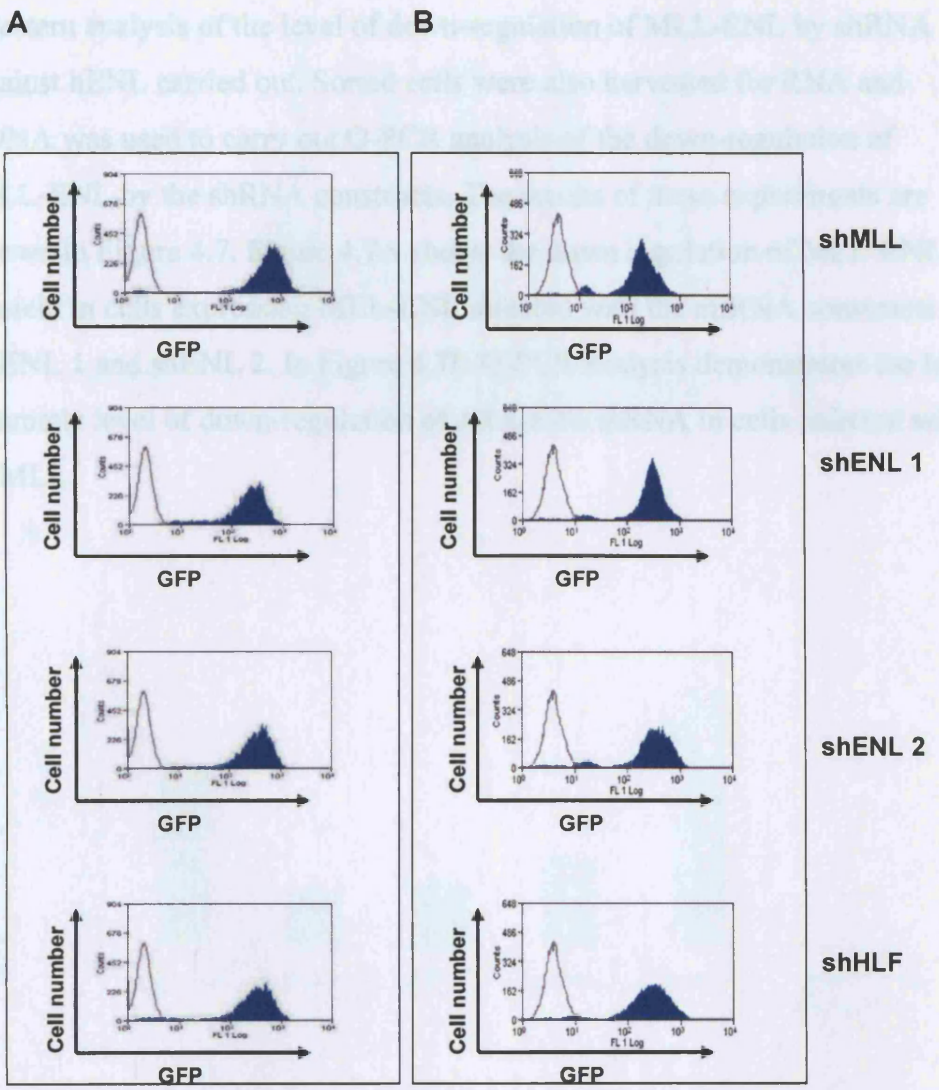


Figure 4.6 Expression of GFP in 32DMENL cells infected with pSFFV-GFP-miR-shRNA constructs.

Panel **A** shows expression of GFP over 14 days reflecting stable expression of the miRNA constructs shENL 1, shENL 2, shMLL and shHLF labelled. Panel **B** shows high expression of GFP in cells infected with each of the constructs and then sorted for GFP expression and expanded for 24 hours.

32MENL cells sorted for GFP expression were harvested for protein and Western analysis of the level of down-regulation of MLL-ENL by shRNA against hENL carried out. Sorted cells were also harvested for RNA and cDNA was used to carry out Q-PCR analysis of the down-regulation of MLL-ENL by the shRNA constructs. The results of these experiments are shown in Figure 4.7. Figure 4.7A shows the down regulation of MLL-ENL protein in cells expressing MLL-ENL infected with the miRNA constructs shENL 1 and shENL 2. In Figure 4.7B Q-PCR analysis demonstrates the less dramatic level of down-regulation of *MLL-ENL* mRNA in cells infected with shMLL.

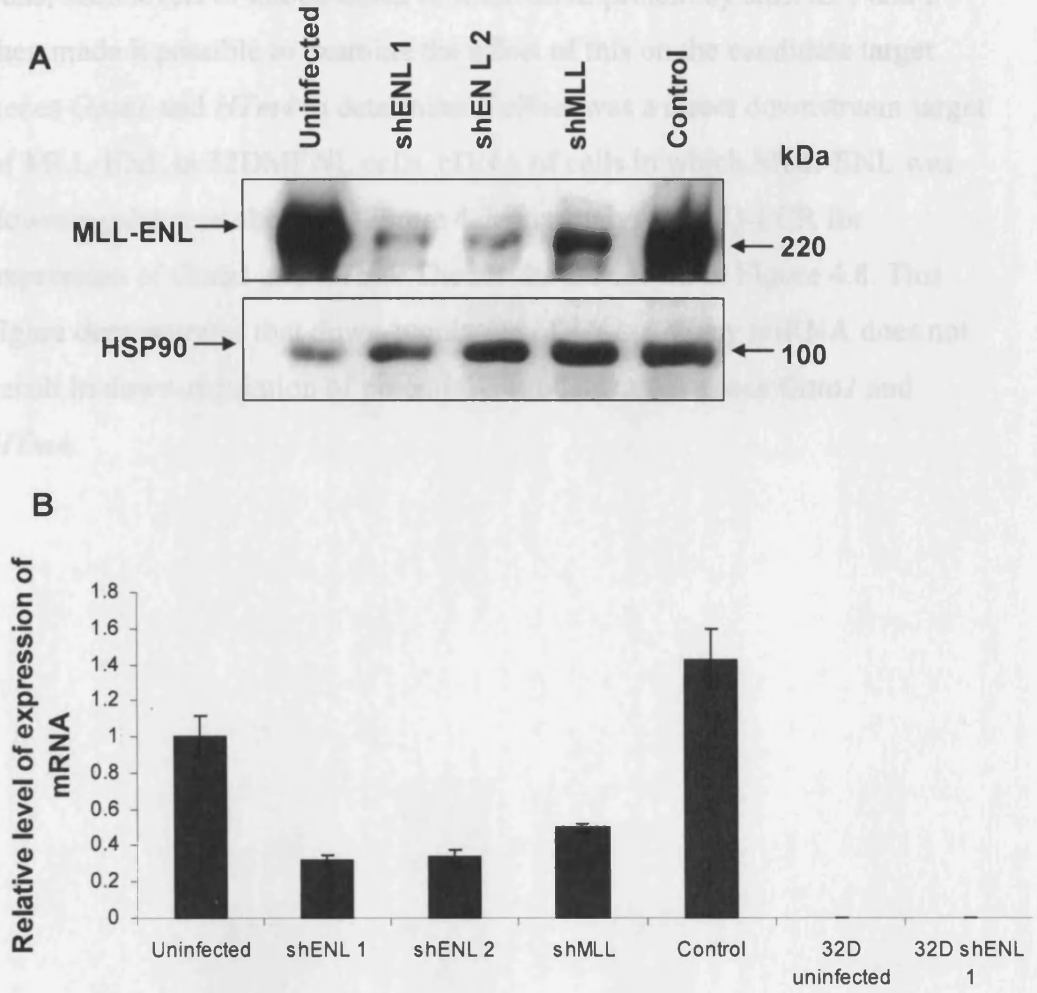


Figure 4.7 Expression of MLL-ENL following infection with shRNA against human *ENL*.

A shows Western analysis of MLL-ENL protein in cells expressing MLL-ENL infected with shENl 1 and shENL 2. Parental 32D cells uninfected and infected with shENL 1 are shown. **B** shows Q-PCR demonstrating the level of expression of *MLL-ENL* mRNA in these cells.

In the absence of an inducible system of MLL-ENL expression in 32DMENL cells, such levels of knock-down of MLL-ENL protein by shENL 1 and 2 then made it possible to examine the effect of this on the candidate target genes *Gata1* and *HTm4* to determine if either was a direct downstream target of MLL-ENL in 32DMENL cells. cDNA of cells in which MLL-ENL was down-regulated as shown in Figure 4.7 was analysed by Q-PCR for expression of *Gata1* and *HTm4*. The results are shown in Figure 4.8. This figure demonstrates that down-regulation of *MLL-ENL* by miRNA does not result in down-regulation of potential candidate target genes *Gata1* and *HTm4*.

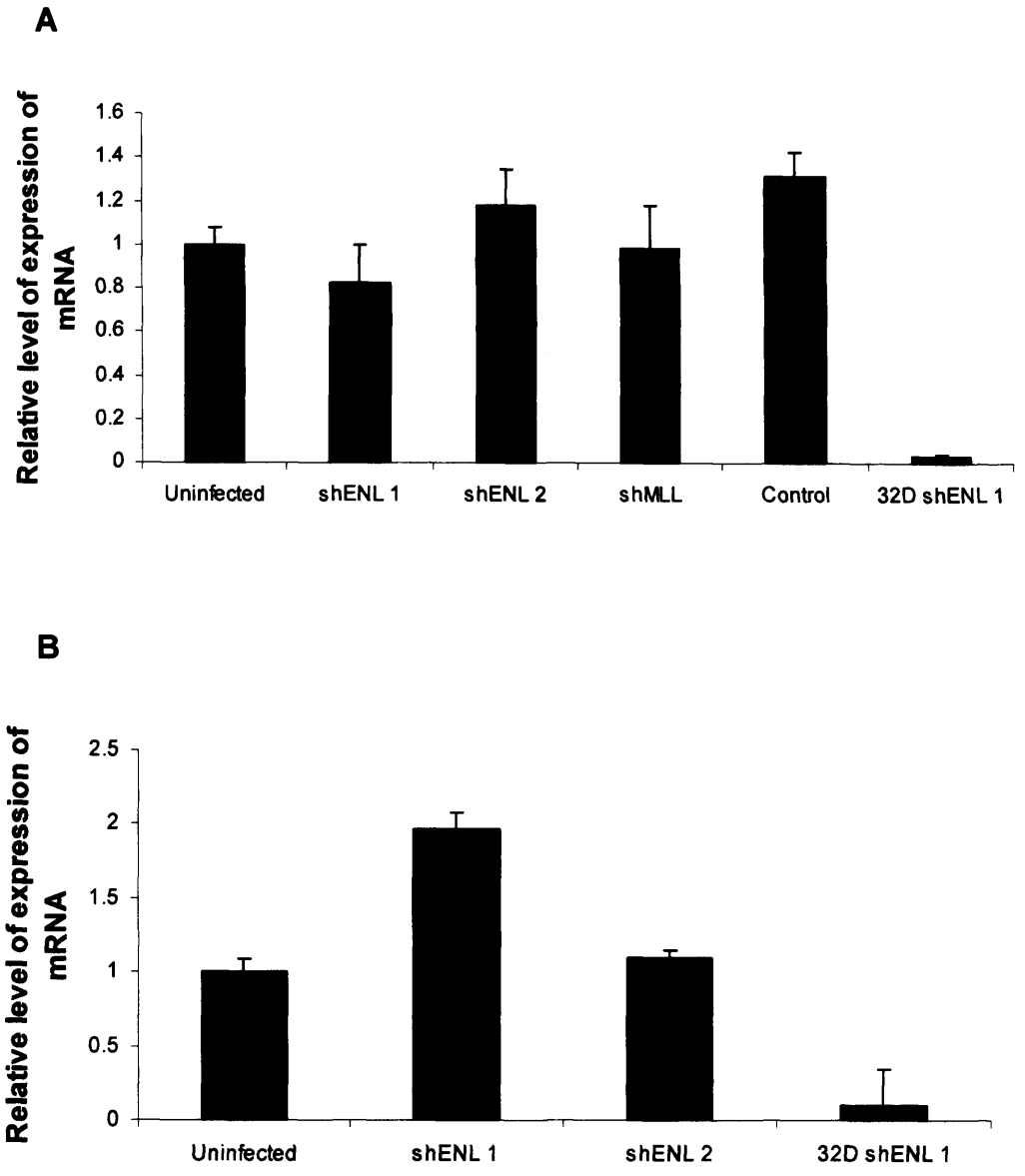


Figure 4.8 Q-PCR of *Gata1* and *HTm4* in 32DMENL cells following knock-down of MLL-ENL.

A shows *Gata1* and **B** shows *HTm4* mRNA levels in 32DMENL cells in which there is down-regulation of MLL-ENL by shENL 1 and shENL 2 as seen in Figure 4.7.

The figure shows that neither *Gata1* nor *HTm4* are down-regulated in the presence of the achieved levels of MLL-ENL knock-down.

The results may be interpreted in the following ways:

- saturation of GATA1/MLL-ENL or HTm4/MLL-ENL binding sites by the MLL-ENL still resulted due to insufficient levels of down-regulation of the fusion protein
- MLL-ENL binds to GATA1 or HTm4 to initiate its expression, but that continued or maintained levels of either protein are not dependent on MLL-ENL and are not affected by down-regulation of the fusion protein
- neither *Gata1* nor *HTm4* is a direct target of MLL-ENL
- *HTm4* may require the high-affinity menin-binding domain of MLL to more tightly regulate its expression in the presence of MLL fusion proteins. This behaviour would be similar to that of other cell cycle regulator genes. As HTm4 regulates HSC cycling, its expression may be more clearly regulated in these cells compared to 32Dcl3.

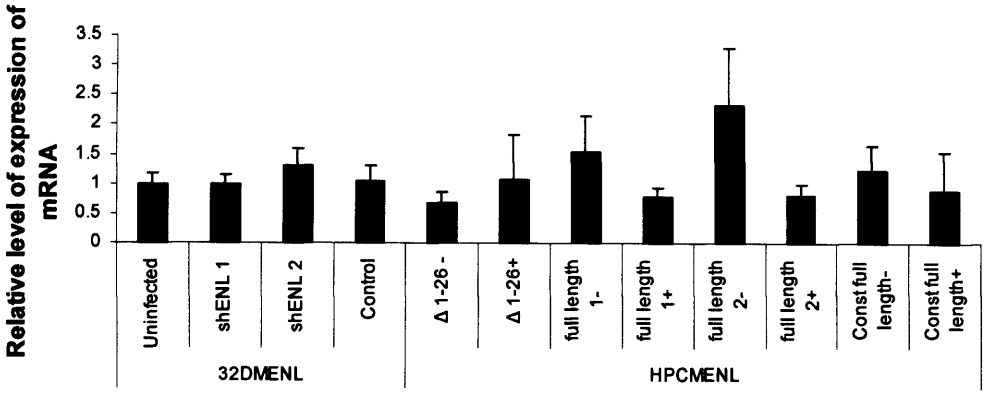
In order to determine whether *HTm4* was target of MLL-ENL, its expression was studied in a more appropriate cell type, under tightly controlled levels of MLL-ENL expression, and in the presence of MLL-ENL expressing the high-affinity Menin-binding domain. Q-PCR analysis of *HTm4* expression was determined in immortalized HPC expressing full-length MLL-ENL ($HPCMENL^{full\text{-}length}$) whose expression was proven in the laboratory to be regulated in a tetracycline-inducible manner so that it was silenced in the presence of doxycycline.

HTm4 is down-regulated in immortalized primary HPC when full length MLL-ENL containing the Menin-binding domain is turned off. A system of tightly regulated, tetracycline-inducible expression of MLL-ENL in HPC has been generated in our laboratory (Horton, *et al* 2005). These HPC were

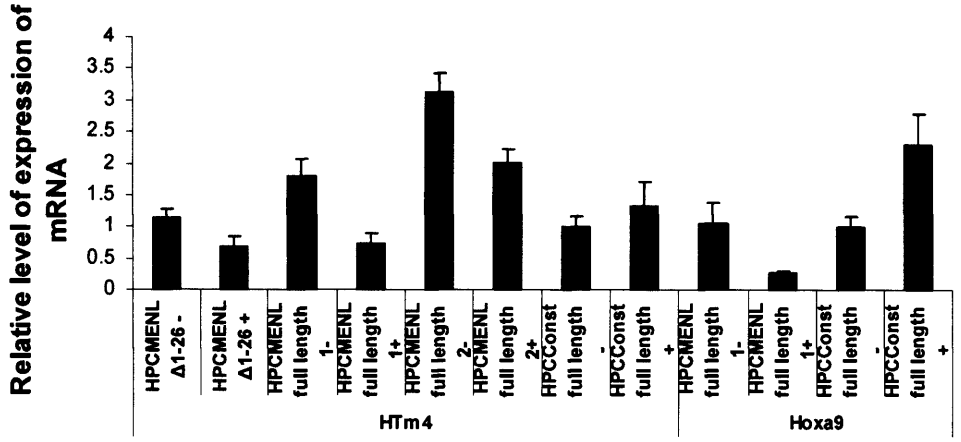
infected with pMSCV-neo-MLL-ENL$^{\Delta 1\text{-}26}$ (HPCMENL$^{\Delta 1\text{-}26}$). These HPC were dependent on continued MLL-ENL expression for their sustained proliferation and inhibition of differentiation. The high-affinity Menin-binding domain is required by MLL, and possibly MLL fusion proteins, to effectively regulate expression of its target genes (Milne, *et al* 2005a). The MLL-ENL construct expressed in the HPCMENL $^{\Delta 1\text{-}26}$ and in 32DMENL cells lacked the first 26 amino-acids of MLL, which contained this Menin-binding domain. Therefore, HPC have also been generated that are conditional for the expression of full-length MLL-ENL (HPCMENL$^{\text{full-length}}$) and these cells also have a similar requirement for MLL-ENL-dependent proliferation. Control HPC that were not tetracycline-responsive and which constitutively expressed full-length MLL-ENL (HPCconst$^{\text{full-length}}$) were also generated (Horton et al., manuscript in preparation).

cDNA was derived from both HPCMENL$^{\Delta 1\text{-}26}$ cell lines, and HPCMENL$^{\text{full-length}}$ cell lines, as well as HPCconst$^{\text{full-length}}$, in the presence and absence of doxycycline. Q-PCR analysis for *HTm4* was carried out to determine if *HTm4* was regulated by MLL-ENL in HPCMENL$^{\text{full-length}}$ containing the Menin-binding domain. The results in Figure 4.9 show that *HTm4* is down-regulated in HPC in the absence of MLL-ENL (when doxycycline is present) in HPCMENL$^{\Delta 1\text{-}26}$ which contains only the low-affinity Menin-binding domain. However, this regulation is seen more greatly in HPCMENL$^{\text{full-length}}$ where *HTm4* is down-regulated in two HPCMENL$^{\text{full-length}}$ cell lines when MLL-ENL is turned off in the presence of doxycycline. *Hoxa9* is known to be down-regulated in the absence of MLL-ENL serving as a positive control. Thus *HTm4* is a candidate target gene of MLL-ENL in HPC, and its regulation by MLL-ENL is likely to require the high-affinity menin binding domain of the MLL portion of the fusion.

A


Relative level of expression of mRNA
3.5
3
2.5
2
1.5
1
0.5
0
Uninfected
shENL 1
shENL 2
Control
Δ 1-26 -
Δ 1-26+
full length 1-
full length 1+
full length 2-
full length 2+
Const full length-
Const full length+
32DMENL
HPCMENL

B


Relative level of expression of mRNA
4
3.5
3
2.5
2
1.5
1
0.5
0
HPCMENL Δ1-26 -
HPCMENL Δ1-26 +
HPCMENL full length 1-
HPCMENL full length 1+
HPCMENL full length 2-
HPCMENL full length 2+
HPCConst full length -
HPCConst full length +
HPCMENL full length 1-
HPCMENL full length 1+
HPCConst full length -
HPCConst full length +
HTm4
Hoxa9

C

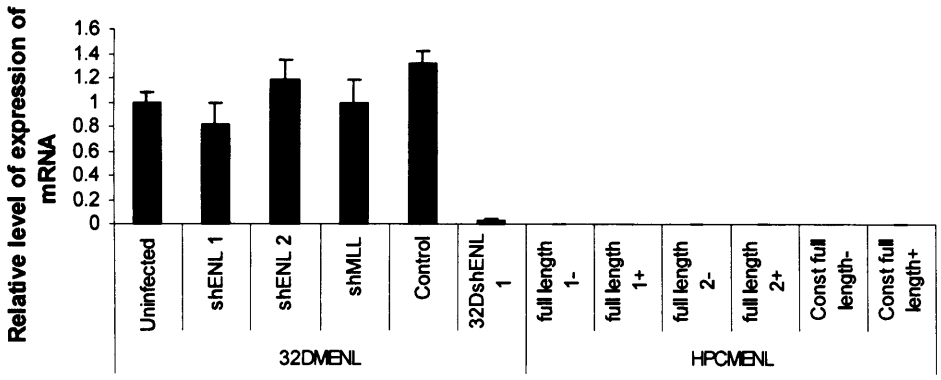


Figure 4.9 HTm4 is regulated in primary cells immortalised with full-length MLL-ENL containing the Menin-binding domain.

This figure shows Q-PCR analysis of **A** *HTm4* mRNA levels in primary cells transduced with inducible HPCMENL$^{\Delta 1\text{-}26}$ lacking the Menin-binding domain, and **B** *HTm4* mRNA levels in HPCMENL$^{\text{full-length}}$ containing the Menin-binding domain, when MLL-ENL expression is turned off in the presence of doxycycline (+) and turned on in the absence of doxycycline (-) **C** shows *Gata1* expression in HPCMENL$^{\text{full-length}}$ in the presence and absence of doxycycline.

4.5 Discussion

4.5.1 The prospective role of new validated targets of MLL-ENL in leukaemogenesis

The candidate targets identified by microarray analysis are known to have a role in normal and malignant haematopoiesis and their further analysis in 32Dcl3 cells and in primary haematopoietic cells may identify them as contributing toward the leukaemic phenotype. A brief description of each candidate gene is described below.

Htm4

This is a novel protein, expressed in human haematopoietic cells and bone marrow, and acts as a regulator of the haematopoietic cell cycle by binding directly to KAP to modulate the level of phosphorylation of CDK2 (Donato, *et al* 2002). *HTm4* binds via its C-terminal region to the complex formed between KAP and CDK2 to stimulate phosphatase activity and modulate KAP activity within the KAP-CDK2 complex. It belongs to the MS4 (membrane spanning 4) protein group with a distinctive four-transmembrane structure, and is also expressed in developing and adult murine haematopoietic and central nervous systems (Kutok, *et al* 2005). Of interest is the finding that HTm4 has a role in mediating HSC quiescence by modulating G1 to S cell cycle transition, and is tightly regulated during normal haematopoietic stem cell differentiation, and its exogenous expression in U937 cells leads to G0/G1 arrest. Aberrant expression of this gene in the presence of an oncogene may therefore result in abnormal regulation of cell cycle in cells that may then develop a leukaemogenic potential.

Gata1

Critical in multilineage homeostasis, GATA1 binds sites in the promoters and enhancers of erythroid-specific genes and is essential for erythroid and megakaryocyte (MegE) development (Friedman 2002). Ectopic GATA1 prevents normal lymphoid and granulocyte/macrophage differentiation, re-directing them toward MegE commitment (Iwasaki, *et al* 2003). Ectopic GATA1 in 416B (a myeloid cell line capable of megakaryocyte and granulocyte differentiation) induces megakaryocyte development (Visvader, *et al* 1995), and this may be relevant to the finding that the megakaryocyte marker CD41 is up-regulated in 32DMENL cells where GATA1 is highly expressed. In addition, GATA1 induces apoptosis in proB and myelomonocytic cells which is not abrogated by BCL2 and this is again considered in relation to the profile of apoptotic sensitivity seen in 32DMENL cells in the current study.

Ang1

Angiopoietin is a growth factor involved in angiogenesis. Angiopoietin-1 (ANG1) and angiopoietin-2 (ANG2) are ligands for the endothelial cell-specific receptor tyrosine kinase TIE2, a transcription factor in early haematopoiesis (Davis, *et al* 1996). The interaction between haematopoietic cells and endothelial cells is important in normal haematopoiesis as well as leukaemogenesis. The cell cycle status of the HSC is dynamically controlled by the microenvironment, and the interaction of TIE2 and its ligand ANG1 leads to tight adhesion of HSC to stromal cells, resulting in maintenance of long-term repopulating activity of HSC (Arai, *et al* 2004, Hirao, *et al* 2004). Thus, the TIE2/ANG1 signalling pathway plays a critical role in the maintenance of HSC in a quiescent state in the BM niche, and its deregulation may cause aberrant proliferation or survival of primary cells.

Il-6

Interleukin-6 (IL-6) is a lineage-specific transcription factor which stimulates granulopoiesis via receptor signaling (Liu, *et al* 1997). The transcription factor C/EBPa is required for myeloid differentiation, and C/EBPa $^{-/-}$ mice show loss of IL-6 receptor and lack of IL-6 responsive colony forming units. Previously such mice have been shown to lack the G-CSF receptor. The addition of soluble IL-6 receptor and IL-6 rescues these mice in their ability to form granulocytes. Thus IL-6 and G-CSF, and possibly other cytokine receptors, are important for the block in granulocyte differentiation observed in vivo in C/EBPa-deficient mice (Zhang, *et al* 1998). Deregulation of IL-6 may result in aberrant myeloid differentiation, and may be implicated in the macrophage phenotype seen in 32DMENL cells.

Apo J

APO J is a complex heterodimeric glycoprotein up-regulated during tumorigenesis, as well as during cell injury or death. Secreted APO J is anti-apoptotic in the presence of increased b-Myb but over-expression sensitises malignant cell lines to cytotoxic-induced apoptosis (Trougakos, *et al* 2005). Increased apoptosis is also seen in neuroblastoma cells over-expressing secreted APO J and co-cultured with an APO J- blocking antibody (You, *et al* 2003). In stress situations, the nuclear protein is pro-papoptotic and cell specific (Trougakos, *et al* 2005). Thus, APO J may have a role in the phenotype of increased apoptotic sensitivity seen in 32DMENL cells.

Mpo

Myeloperoxidase (MPO) is a lysosomal haemoprotein located in the azurophilic granules of polymorphonuclear (PMN) leukocytes and monocytes. MPO is located in the nucleus as well as in the cytoplasm. Intranuclear MPO may help to protect DNA against damage resulting from oxygen radicals produced during myeloid cell maturation and function (Borregaard and Cowland 1997). MPO gene was translocated to

chromosome 15 in all cases of acute promyelocytic leukemia (subtype M3), MPO may be pivotal in the pathogenesis of APL (Weil, *et al* 1988).

Hba1

The alpha and beta loci determine the structure of the two types of polypeptide chains in the tetrameric adult haemoglobin, HbA, alpha-2/beta-2. Haemoglobin alpha locus-1 (*HBA1*) encodes the alpha polypeptide chain. This gene was highlighted in a gene expression analysis of FL5.12 pro B cells where apoptosis was induced either following cytotoxic treatment or following IL-3 withdrawal (Brachat, *et al* 2000). Over-expression of HBA1 was found to be pro-apoptotic after IL-3 withdrawal, similar to BAX over-expression and associated with increased caspase activity. This gene was therefore further validated in view of the increased apoptotic sensitivity of 32DMENL cells following IL-3 withdrawal.

4.5.2 The role of Menin in the expression of target genes of MLL fusion proteins

The role of Menin in its association with MLL as part of a histone methyltransferase complex to regulate Hox gene expression has been described in Chapter 1 together with its implication in the aberrant regulation of Hox genes by MLL-fusion proteins. In addition, Menin is a known regulator of the CDKI genes p27Kip1 and p18Ink4c (Milne, *et al* 2005a) and the association of Menin with MLL fusion proteins may similarly result in de-regulation of such CDKI. The expression of p27Kip1 is increased following inducible MLL-AF4 in a lymphoid leukaemia cell line, and these cells show slower growth without increased apoptosis compared to control (Xia, *et al* 2005). In this study ChIP assays suggested that p27Kip1was a direct target of MLL-AF4 as well as of WT MLL and increased expression by MLL-AF4 may be responsible for the observed reduction in lymphoid cell cycling (Xia, *et al* 2005). Suppression of cell proliferation among HSC harbouring MLL fusion genes may have implications for increased

chemotherapy resistance in these poor prognosis infant leukaemias with a high relapse rate. Alterations of the tumour suppressors p15Ink4a and p16Ink4b have also been associated with MLL fusions in both myeloid and lymphoid leukaemia cells lines (Ohnishi, *et al* 1997) (Maloney, *et al* 1997).

4.5.3 A model of HTm4 as a target gene of MLL-ENL whose activation by the fusion protein is mediated by Menin

In this study, *HTm4* has been identified as a potential target of MLL-ENL, being significantly up-regulated by microarray analysis in the presence of constitutive over-expression of MLL-ENL in 32Dcl3 cells. *HTm4* has been previously identified as a novel human haematopoietic cell cycle regulator of G1/S phase cell cycle transition, and is tightly regulated during HSC differentiation to maintain their quiescent state (Donato, *et al* 2002). This study has demonstrated that *HTm4* is up-regulated following Q-PCR analysis in seven of eight 32D clones constitutively expressing MLL-ENL, and up to 160-fold in these cells. However, following down-regulation of MLL-ENL by shRNA against human ENL in a single 32DMENL clone, no down-regulation of *HTm4* was observed.

Reasons for the initial high expression but failed down-regulation of *HTm4* are:

- the levels of shRNA inhibition of MLL-ENL are not great enough to prevent the maintenance of HTm4 expression if *HTm4* binding sites are already saturated by remaining levels of MLL-ENL protein.
- MLL-ENL may be responsible for the initiation but not the maintenance of HTm4 expression. As 32D cells are more differentiated, they may not have the same requirement for *HTm4* as HPC in order to maintain a quiescent state (Donato, *et al* 2002).

The subsequent down-regulation of *HTm4* in a tightly regulated system of MLL-ENL expression supports the first possible explanation to be more likely.

The results of *HTm4* expression in HPC transduced with truncated and full-length MLL-ENL constructs suggest a role for Menin in mediating transcription of *HTm4* by MLL-ENL in primary cells.

The pTRE-MLL-ENL and pMSCV-neo-MLL-ENL$^{\Delta 1\text{-}26}$ constructs used lacked the first 26 amino acids which may contain domains that are necessary for direct and specific binding of MLL-ENL to its targets. One such domain is the Menin binding domain, and the high affinity domain is contained in first 35 amino acids of MLL which is therefore absent in the truncated construct. The low affinity domain is present in this construct at amino acids 640-1251. (Yokoyama, *et al* 2005). The pTRE-MLL-ENL was used in the transfection of 32Dcl3 cells. It may be that different cells may have different requirements for Menin in the regulation of the target gene by MLL-ENL. Thus a truncated MLL-ENL construct may be sufficient to induce expression of *HTm4* via its low affinity domain in 32DMENL and HPCMENL$^{\Delta 1\text{-}26}$, but a specific requirement of Menin may exist to allow greater specific binding and regulation of *HTm4* as seen in HPCMENL$^{\text{full-length}}$.

In order to test the cell specificity of *HTm4* regulation, the expression was examined in HPC expressing MLL-ENL where there was tight inducible regulation of the fusion gene. MLL-ENL expression is abrogated in the presence of doxycycline in HPCMENL cells via the Tetoff system. In HPCMENL$^{\Delta 1\text{-}26}$, compared to 32DMENL cells, *HTm4* expression was regulated by MLL-ENL. This is possibly due to the specific requirement of *HTm4* for cell cycle regulation in this cell type compared to 32D cells.

Furthermore, *HTm4* was regulated to an even greater extent in HPCMENL$^{full-length}$ expressing the full length fusion protein including the Menin binding domain, with *HTm4* being down-regulated when MLL-ENL was turned off. These results suggest a specific requirement for Menin in these cells in order to more effectively regulate *HTm4* by MLL-ENL. MLL-ENL may function to induce cell cycle inhibition of HPC via its up-regulation of *HTm4* in order to inhibit cell proliferation. Either this could be a mechanism by which HPC harbouring MLL fusions evade chemotherapy, allowing them to emerge later to cause relapse of disease, or it allows an environment where a HSC harbouring an MLL fusion is more able to acquire another mutation in order to escape such proliferation inhibition, and such a cell would be a candidate for initiation of leukaemogenesis.

The possibility that a normal HPC may progress to a leukaemic stem cell has been suggested by a study where MLL-AF9 immortalises a committed progenitor to create a leukaemic stem cell as a result of re-activation of a subset of genes normally only expressed in HSC (Krivtsov, *et al* 2006). In this respect *HTm4* is a candidate gene that may be involved in such a pathway leading to a cancer stem cell, and makes it potentially a valuable target in the eradication of the generation of stem cells which initiate and maintain leukaemia, such a strategy being more likely to achieve effective cure rather than the reduction in tumour burden alone. *HTm4* is known to regulate normal HSC quiescence (Donato, *et al* 2002), is found to be highly expressed in a granulocyte macrophage progenitor cell in this study, and its regulation in HPC over-expressing MLL-ENL in this study correlates with the presence of the high-affinity Menin-binding domain known to associate with MLL fusion proteins to regulate other CDKI and *Hox* gene targets (Milne, *et al* 2005a) (Yokoyama, *et al* 2005).

4.5.4 Further work

Analysis of the candidate target genes identified by microarray analysis for any role in deregulating the proliferation, survival and differentiation of primary haematopoietic cells would identify them as contributors to the leukaemic phenotypedue to MLL-ENL expression. *HTm4* will be studied further as it has been shown to be down regulated following shRNA inhibition of *MLL-ENL*.

The ability of *HTm4* to immortalise and transform myeloid and lymphoid progenitor cells, either alone or in co-operation with other potential oncogenes, will be assessed using colony-forming assays in methylcellulose where the number of colonies formed during successive rounds of re-plating reflects the self-renewal and proliferative capacity of the HPC (Lavau, *et al* 1997). *HTm4* will be introduced into HPC derived from murine bone marrow or foetal liver by retroviral transduction. This work will continue in the laboratory by another member of the group, and will identify any functional role that *HTm4* might have in leukaemogenesis.

5 MLL-ENL acts in a gain-of-function manner to immortalize *Mll* deleted haematopoietic progenitor cells

Very different human pathological conditions arise from either gain- or loss-of-function mutations of the same gene. For example, gain-of-function mutations in the *RET* gene results in growth factor independent signalling of the RET protein leading to the syndrome of multiple endocrine neoplasia type 2, where familial medullary thyroid carcinoma develops as part of one of the disease subtypes. However, a non-functional protein results from a loss-of-function mutation of the *RET* gene leading to abnormal development of enteric nerves to cause Hirschprung's disease (Lesueur, *et al* 2006, Sijmons, *et al* 1998). In relation to human leukaemia, a loss-of-function mutation of the CREB binding protein (*CBP*) gene results in a characteristic syndrome of cardiac anomaly, broad thumbs, big toes and typical facies (Rubinstein-Taybi syndrome). However, fusion proteins formed following translocation of the *CBP* gene on chromosome 16 to form MOZ-CBP [t(8;16)] and MLL-CBP [t(11;16)] are associated with AML, either *de novo* or following previous anticancer treatment in the case of MLL-CBP (Taki, *et al* 1997). Such fusion proteins are thought to contribute to haematological malignancy through a gain-of-function mechanism, perhaps accompanied by the inactivation of the un-rearranged *CBP* gene (Taki, *et al* 1997).

Models have been proposed relating to the normal function of MLL in the presence of MLL fusion genes (see (Ernst, *et al* 2002) for review). A simple gain-of-function model proposes that normal MLL function is altered to cause over-expression of an MLL target gene involved in cell survival or proliferation. A second model proposes that in addition to gain-of-function of one *MLL* allele, the second allele becomes haploinsufficient with resulting

under-expression of a gene normally expressed to negatively regulate survival or proliferation. Finally a dominant-negative model proposes that the *Mll* fusion gene simply interferes with the normal expression by MLL of a gene which negatively regulates cell survival and proliferation.

A model of gain-of-function mediated transformation is thought to occur in the case of leukaemia associated with *MLL* fusion genes, although there is no conclusive experimental proof that this is the case. As described in Chapter 1.5, leukaemia develops in *Mll-Af9* knock-in mice where one copy of *Mll* is replaced by *Mll-Af9*. However, knock out of one copy of *Mll* does not result in leukaemia, which would have been expected if disease was due to *Mll* loss-of-function (Corral, *et al* 1996). Also in support of a gain-of-function mechanism, a number of studies have shown that retroviral insertion of *Mll* fusion genes into HPC transforms cells, and gives rise to leukaemia without disrupting the endogenous *Mll* gene (So and Cleary 2002);(Lavau, *et al* 1997)). On the other hand, loss of one copy of *Mll* in a knock-in model where *Mll* is fused to a short cMYC tag polypeptide does not lead to leukaemia (Corral, *et al* 1996). Furthermore, *Mll-Af9* knock-in mice do not show defects similar to those seen in *Mll* knockout or heterozygote mice (Corral, *et al* 1996);(Dobson, *et al* 1999); (Dobson, *et al* 2000) as would be expected if there was *Mll* loss-of-function in these knock-in mice. These studies imply that MLL fusions act dominantly over endogenous MLL in a gain-of-function manner, rather than by MLL fusions inhibiting endogenous MLL function. The PHD domain of MLL is lost in the fusion protein and binding of Cyp33 to this domain normally represses MLL target genes. Therefore, loss of the PHD domain may change the balance of MLL function towards transactivation in the fusion protein (Fair, *et al* 2001).

However, a possible role for wild-type (WT) *Mll* in contributing to transformation is suggested by studies which show high degrees of *Mll* gene amplification by fluorescent *in situ* hybridization (FISH) in MDS and

leukaemia (Papenhausen, *et al* 2005). In addition, studies show that WT MLL is bound to the promoters of *Hoxa9* and *Meis1* in the presence of the fusion proteins MLL-ENL-ER and dimerised MLL-FKBP, suggesting that WT MLL may be recruited by these fusion proteins to contribute to transformation, or that MLL may simply bind to target loci (Milne, *et al* 2005b).

In order to determine the dependence of transformation on WT MLL, the MLL fusion protein MLL-ENL was expressed in *Mll*$^{-/-}$ HPC. The ability of MLL-ENL to immortalise such *Mll* knockout (KO) HPC would provide substantial evidence for a gain-of-function mechanism of MLL-ENL leading to leukaemia.

Previous *Mll* knockout embryos have demonstrated abnormalities in haematopoiesis with reduced cellularity and colony forming capacity in *Mll* knockout yolk sac and foetal liver HPC (Hess, *et al* 1997, Yagi, *et al* 1998)). Knockout HPC from the AGM of *Mll* deficient mice were also unable to reconstitute the bone marrow of irradiated mice compared to WT controls (Ernst, *et al* 2004a). However, because homozygous deletion of *Mll* results in embryonic lethality, a knockout mouse model was generated in our laboratory in which deletion of exons 9 and 10 of both alleles was achieved in a conditional manner (McMahon, *et al* 2007 manuscript submitted). These embryos died at E12.5-16.5, and analysis of E12.5-E14.5 foetal liver cells showed defects in the haematopoietic stem and progenitor pools, including reductions in LT-HSC and ST-HSC number. In the conditional model, LoxP sites were inserted flanking exons 9 and 10 of the *Mll* allele (floxed allele), and these sites were subject to recombination in the presence of the enzyme Cre recombinase. The breeding of mice carrying a floxed *Mll* allele to mice carrying the *Vav-Cre* transgene resulted in a further conditional knockout (cKO) model in which *Mll* was deleted in cells of the haematopoietic system only, as the *Vav* promoter was only expressed in haematopoietic cells, and

this allowed the effects of *Mll* deletion on post-natal haematopoiesis to be studied (McMahon, *et al* 2007 manuscript submitted). Although normal numbers of cells appeared in the bone marrow, spleen and thymus, haematopoietic function was compromised with reduced numbers of pre-B and myeloid colonies seen in culture, and bone marrow cells were unable to compete with WT cells in competitive reconstitution assays. HPC from knockout foetal livers and from conditional knockout adult bone marrow were utilized to examine the effect of MLL-ENL on HPC lacking both *Mll* alleles.

5.1 MLL-ENL immortalizes murine $Mll^{+/+}$ foetal liver and adult bone marrow haematopoietic progenitor cells

Previous models of MLL fusion leukaemia, in which HPC enriched in stem cells were transduced with retrovirally-delivered *Mll* fusion gene constructs, have been described in Chapter 1. In these studies both WT alleles of *Mll* are present in the transduced HPC.

In this study, HPC were isolated from E13.5 foetal liver or adult bone marrow and HPC enriched for stem cells by depleting $Ter119^+$ foetal liver cells or Lin^+ BM cells. These HPC were then transduced with the pMSCV-neo-MLL-ENL$^{full\text{-}length}$ construct shown in Figure 2.4. The results of colony forming assays are shown in Figure 5.1 and Figure 5.2 and demonstrate that MLL-ENL immortalizes $Mll^{+/+}$ HPC. Figure 5.1A shows that large numbers of dense colonies continued to plate into the eighteenth round, and were macroscopically typical of myeloid colony forming units (CFU), demonstrating a high capacity for renewal of the haematopoietic progenitors. Figure 5.1B shows cytospin analysis of the cells showed blast-like morphology, and immunophenotype of $Mac1^+Gr1^+c\text{-}Kit^{low}$, suggesting cells at an immature stage of myeloid differentiation (data not shown).

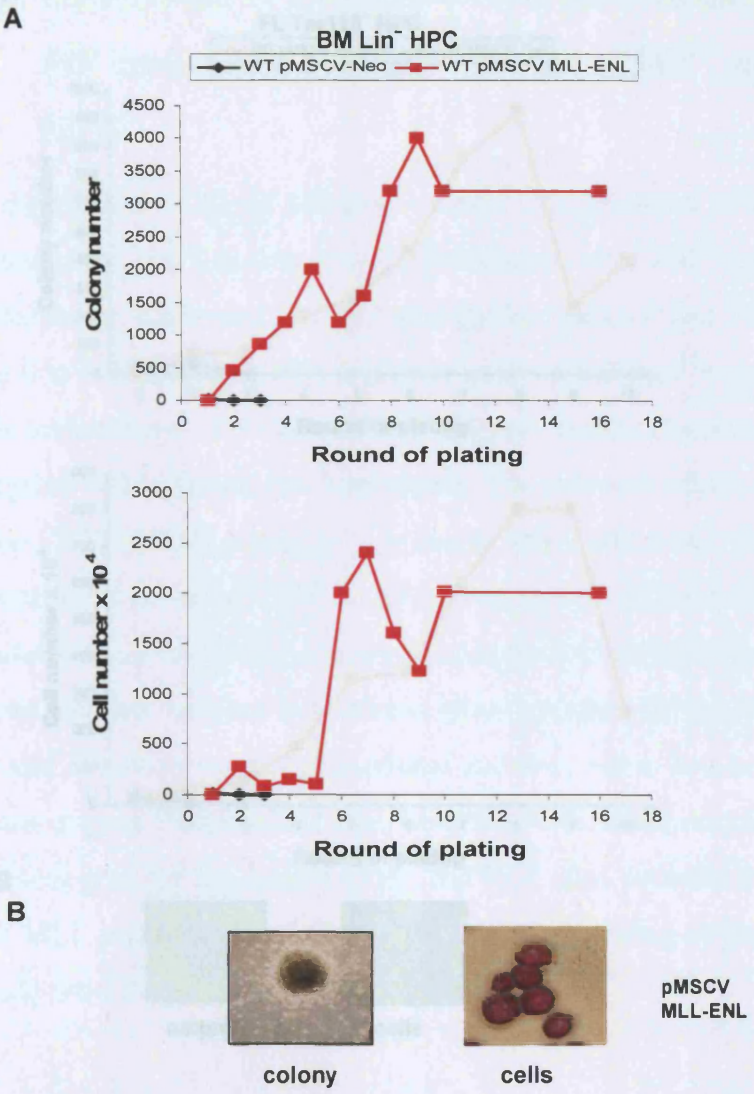


Figure 5.1 Colony and cell number following expression of MLL-ENL in wild type adult murine haematopoietic cells

A shows the number of colonies formed, and number of cells per 10^4 cells plated following serial re-platings of Lineage depleted adult murine HPC transduced with pMSCV-neo- MLL-ENL$^{full\text{-}length}$.

B shows the typical colony and cellular morphology of WT adult HPC immortalised by pMSCV-neo- MLL-ENL$^{full\text{-}length}$. Cells were visualised by cytospin preparation followed by May-Grunwald-Giemsa staining (original magnification x 400).

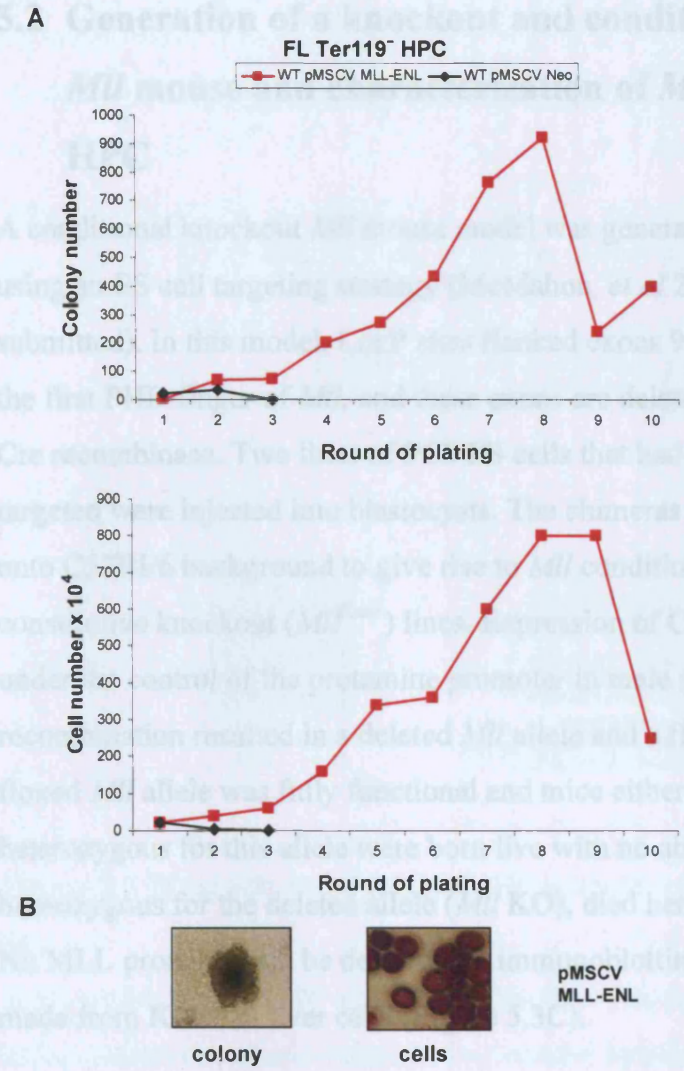


Figure 5.2 Colony and cell number following expression of MLL-ENL in wild type foetal murine haematopoietic cells.

A shows the number of colonies formed, and number of cells, per 10^4 cells plated following serial re-platings of Ter-119 depleted E13.5 foetal murine HPC transduced with pMSCV-neo- MLL-ENL$^{full\text{-}length}$.

B shows the typical colony and cellular morphology of WT foetal HPC immortalised by pMSCV-neo- MLL-ENL$^{full\text{-}length}$.

5.2 Generation of a knockout and conditional knockout *Mll* mouse and characterization of $Mll^{+/-}$ and $Mll^{-/-}$ HPC

A conditional knockout *Mll* mouse model was generated in the laboratory using an ES cell targeting strategy (McMahon, *et al* 2007 manuscript submitted). In this model, LoxP sites flanked exons 9 and 10 which encode the first PHD finger of *Mll,* and these exons are deleted in the presence of Cre recombinase. Two lines of PC3 ES cells that had been successfully targeted were injected into blastocysts. The chimeras which arose were bred onto C57BI/6 background to give rise to *Mll* conditional ($Mll^{flox/flox}$) and constitutive knockout ($Mll^{flox/-}$) lines. Expression of Cre recombinase was under the control of the protamine promoter in male spermatocytes, and recombination resulted in a deleted *Mll* allele and a floxed *Mll* allele. The floxed *Mll* allele was fully functional and mice either homozygous or heterozygous for this allele were born live with no abnormalities. $Mll^{-/-}$ mice homozygous for the deleted allele (*Mll* KO), died between E12.5 and E16.5. No MLL protein could be detected by immunoblotting of protein lysates made from KO fetal liver cells (Figure 5.3C).

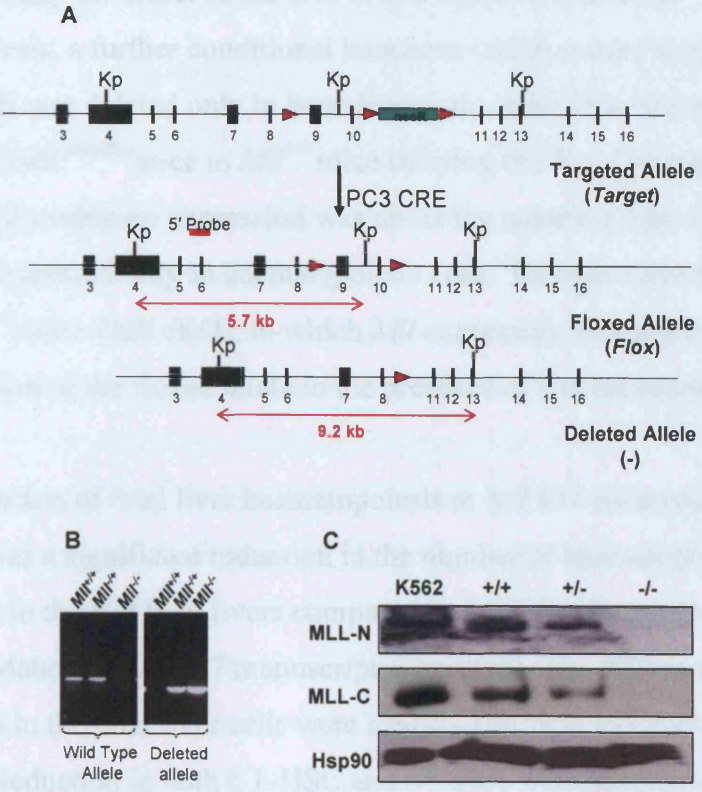


Figure 5.3 Diagram of the targeted *Mll* allele in a conditional mouse cell line.

A shows the targeted *Mll* allele following homologous recombination in ES cells, and the floxed and deleted allele created by recombination of the loxP sites. Red triangles denote LoxP sites. Kp = Kpn1. Also shown are the position of the probe used in Southern Blots and the sizes seen from each allele (Figure courtesy of K. McMahon).

B shows the genotyping by PCR of $Mll^{-/-}$ (KO) embryos. WT allele = 730 bp, Deleted allele = 250bp. (PCR performed by K. McMahon).

C demonstrates the absence of MLL protein in $Mll^{-/-}$ foetal liver by Western blot. Lysates made from foetal liver from $Mll^{+/+}$, $Mll^{+/-}$ and $Mll^{-/-}$ embryos were run alongside K562 lysates as a positive control. MLL N-terminal (MLL-N) and MLL C-terminal (MLL-C) antibodies were used to detect the presence of MLL. An antibody against HSP-90 was used as a loading control. (Western analysis performed by S. Hiew).

In order to study the effect of the loss of *Mll* expression in adult haematopoiesis, a further conditional knockout (cKO) model was developed in which *Mll* was deleted only in haematopoietic cells. This was established by breeding $Mll^{flox/flox}$ mice to $Mll^{+/-}$ mice carrying the *Vav Cre* transgene in which Cre recombinase expression was under the control of the *Vav* promoter, expressed only in haematopoietic cells. This gave rise to $Mll^{-/flox\ VavCre}$ mice (*Mll* cKO), in which *Mll* expression was absent due to recombination of the floxed allele in the presence of Cre recombinase.

Characterisation of fetal liver haematopoiesis in *Mll* KO embryos showed that there was a significant reduction in the number of haematopoietic progenitors in the KO fetal livers compared to $Mll^{+/-}$ (heterozygote, Het), livers (McMahon, *et al* 2007 manuscript submitted). The different HSC populations in the fetal liver cells were analysed by flow cytometry and a significant reduction in both LT-HSC and ST-HSC compartments were seen in the KO fetal livers compared to the Het. Further competitive repopulation assays confirmed that KO fetal liver cells were unable to give rise to either short term or long term reconstitution when compared with *Mll* WT fetal liver cells. Thus, whilst HSC are present in KO fetal livers they were not able to function as self-renewing stem cells when in competition with WT HSC.

Characterisation of the effect of *Mll* deletion on adult haematpoiesis using the cKO model described above showed no difference in any of the major blood cell populations between *Mll* cKO and $Mll^{flox/-}$ (floxed heterozygote, FloxHet) controls. This suggested that *Mll* is not required either to establish normal steady-state haematopoiesis postnatally or for the differentiation of mature haematopoietic cells (McMahon, *et al* 2007 manuscript submitted).

5.3 Retroviral constitutive expression of MLL-ENL and E2A-HLF causes only a transient enhancement of self-renewal in foetal haematopoietic cells

Foetal liver Ter119$^-$ HPC were isolated from E13.5 *Mll*$^{-/-}$embryos and *Mll*$^{+/-}$ littermate controls. The genotype of embryos was confirmed by PCR analysis of DNA extracted from unsorted foetal liver (data not shown). The efficiency of Ter119$^-$ isolation by MACS is shown in Figure 5.4A. These cells were then retrovirally transduced with pMSCV-neo- MLL-ENL$^{full\text{-}length}$ (MLL-ENL), pMSCV-neo (neomycin) and, as a positive control, the known immortalizing oncogene E2A-HLF. The *Mll* gene status has no known relation to the immortalizing potential of E2A-HLF and so could not act to dominantly inhibit MLL function itself. Following spinfection, the cells were transferred to methylcellulose medium M3434 containing recombinant cytokines IL-3, IL-6, SCF and erythropoietin to promote the growth of myeloid lineage cells, and to which GM-CSF was also added. Cells that were resistant to neomycin were assessed for immortalization following serial plating in methylcellulose.

Figure 5.4 shows that *Mll*$^{-/-}$ cells embryos did not undergo immortalization by either MLL-ENL, or E2A-HLF, whereas both oncogenes did immortalize *Mll*$^{+/-}$ HPC. The *Mll*$^{-/-}$ cells formed small, dysmorphic colonies that were not typical of myeloid CFU, up to the fourth round of plating. *Mll*$^{+/-}$ colonies, however, formed typical myeloid CFU and continued to plate beyond the fifth round with high colony and cell numbers.

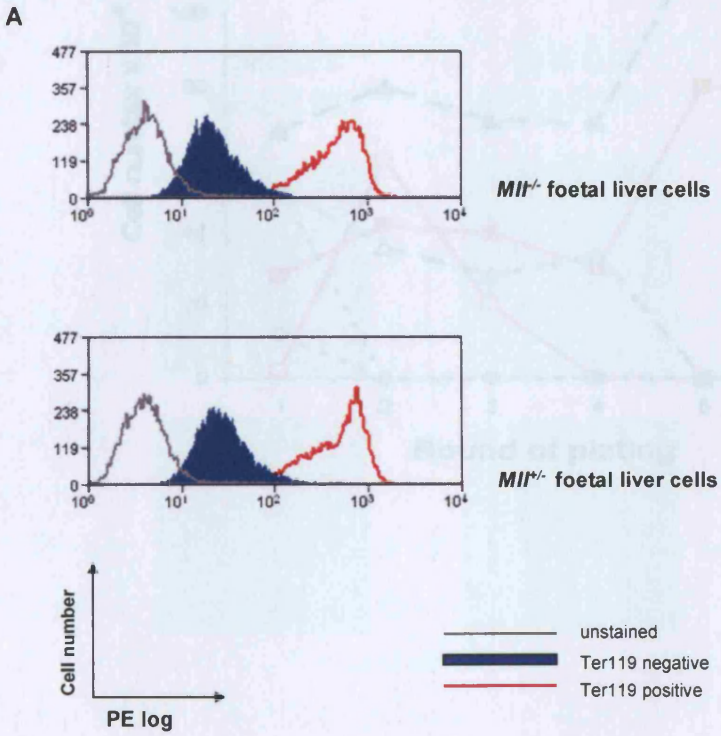
Figure 5.4 Retroviral constitutive expression of MLL-ENL and E2A-HLF causes only a transient enhancement of self-renewal in foetal hamatopoietic cells.

Mll$^{-/-}$ (KO) and *Mll*$^{+/-}$ (Het) foetal liver (FL) HPC were isolated for Ter119^{-} cells and transduced with MLL-ENL, E2A-HLF or neomycin expression constructs, and underwent serial re-plating in methylcellulose.

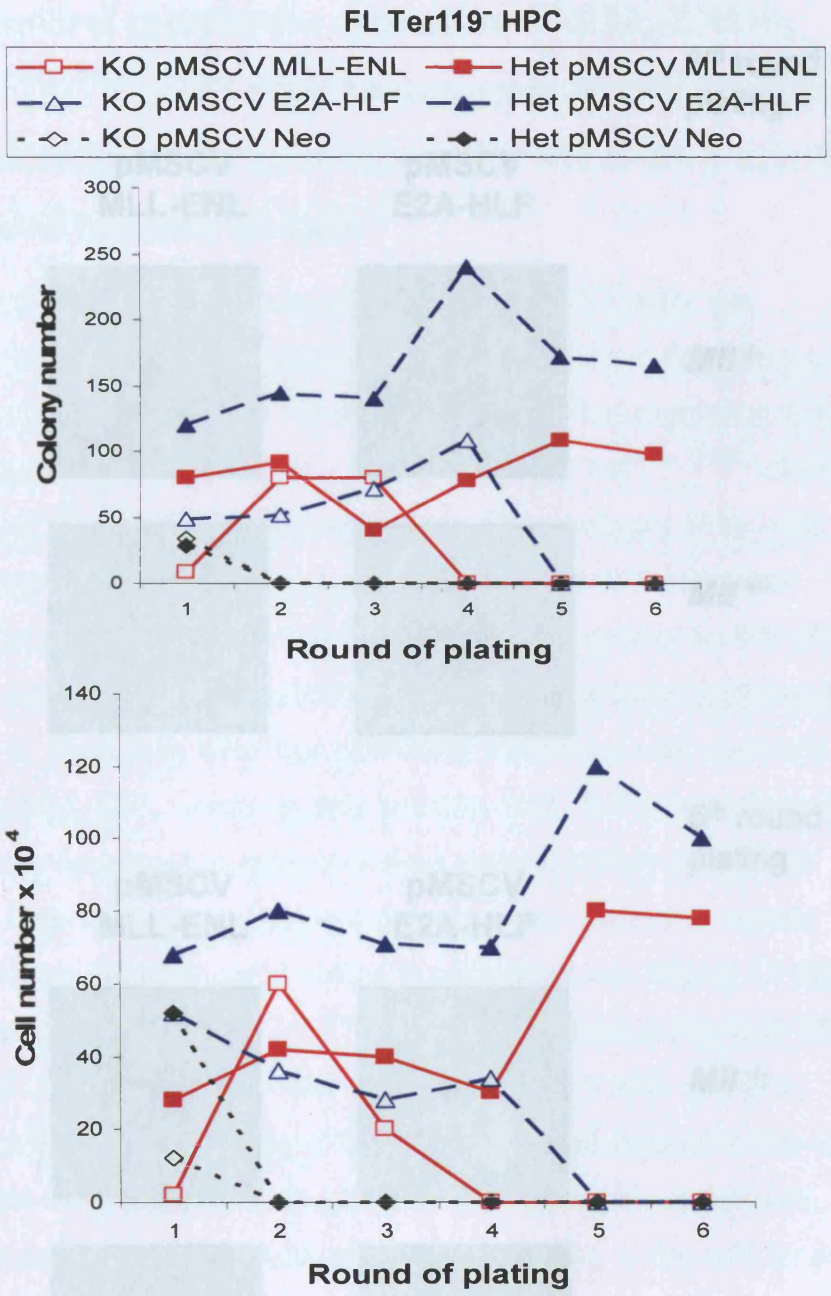
A shows a representative histogram of the typical efficiency of isolation of a Ter119^{-} population by MACS from foetal liver cells. The genotype of *Mll*$^{-/-}$ and *Mll*$^{+/-}$ embryos was confirmed by PCR of DNA extracted from unsorted foetal liver cells (data not shown).

B shows the number of colonies and cells per 10^4 cells plated following serial re-platings from one representative experiment.

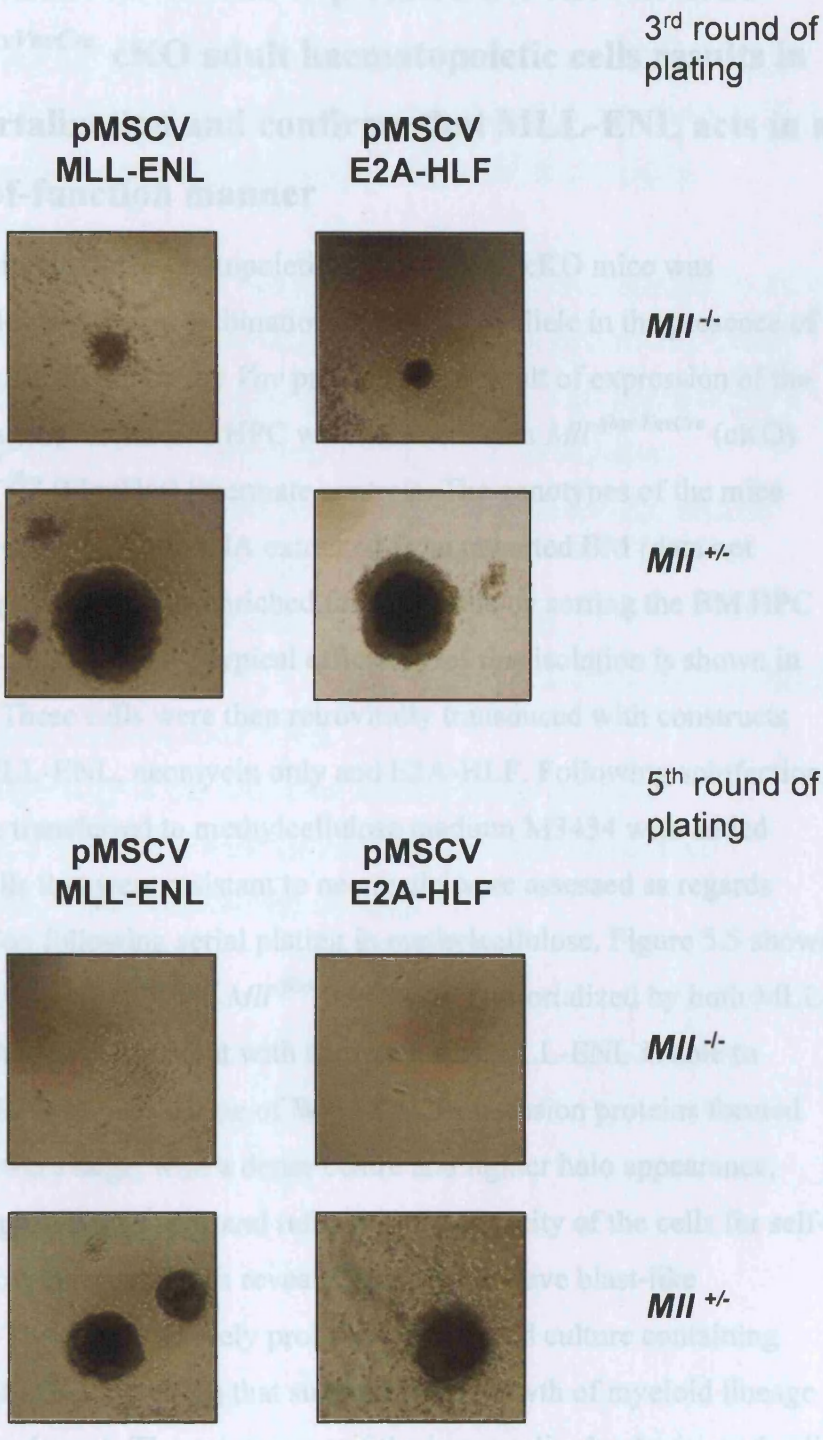
C Typical morphology of *Mll*$^{-/-}$ and *Mll*$^{+/-}$ MLL-ENL and E2A-HLF colonies at the third and fifth round of re-plating (original magnification x40).



B


FL Ter119⁻ HPC
KO pMSCV MLL-ENL
Het pMSCV MLL-ENL
KO pMSCV E2A-HLF
Het pMSCV E2A-HLF
KO pMSCV Neo
Het pMSCV Neo
Colony number
300
250
200
150
100
50
0
1
2
3
4
5
6
Round of plating
Cell number x 10⁴
140
120
100
80
60
40
20
0
1
2
3
4
5
6
Round of plating

C


3rd round of plating
pMSCV MLL-ENL
pMSCV E2A-HLF
Mll -/-
Mll +/-
5th round of plating
pMSCV MLL-ENL
pMSCV E2A-HLF
Mll -/-
Mll +/-

5.4 Retroviral constitutive expression of MLL-ENL in *Mll*$^{-/flox VavCre}$ cKO adult haematopoietic cells results in immortalization and confirms that MLL-ENL acts in a gain-of-function manner

MLL expression in the haematopoietic system of *Mll* cKO mice was abolished following the recombination of the floxed allele in the presence of Cre recombinase driven by the *Vav* promoter as a result of expression of the *Vav Cre* transgene. Adult BM HPC were isolated from *Mll*$^{-/flox\ VavCre}$ (cKO) mice and *Mll*$^{-/flox}$ (FloxHet) littermate controls. The genotypes of the mice were confirmed by PCR of DNA extracted from unsorted BM (data not shown). The population was enriched for stem cells by sorting the BM HPC for a Lin$^-$ population and the typical efficiency of this isolation is shown in Figure 5.5A. These cells were then retrovirally transduced with constructs expressing MLL-ENL, neomycin only and E2A-HLF. Following spinfection, the cells were transferred to methylcellulose medium M3434 with added GM-CSF. Cells that were resistant to neomycin were assessed as regards immortalization following serial plating in methylcellulose. Figure 5.5 shows that both the *Mll*$^{-/flox\ VavCre}$ and *Mll*$^{-/flox}$ HPC were immortalized by both MLL-ENL and E2A-HLF, consistent with the result that MLL-ENL is able to immortalize HPC in the absence of WT MLL. Both fusion proteins formed colonies that were large, with a dense centre and lighter halo appearance, containing high cell numbers, and reflecting the capacity of the cells for self-renewal. Cytospin preparations revealed the cells to have blast-like morphology. These cells actively proliferated in liquid culture containing cytokines (Il-6, IL-3 and SCF) that supported the growth of myeloid lineage cells (data not shown). The appearance of the immortalised colonies and cells was the same for those derived from cells with an *Mll*$^{-/flox\ VavCre}$ or *Mll*$^{-/flox}$ genotype.

Figure 5.5 MLL is not required for the initiation of immortalisation by MLL-ENL.

Mll$^{-/flox\ VavCre}$ and *Mll*$^{-/flox}$ BM HPC transduced with the constitutive MLL-ENL, E2A-HLF, and neomycin expression construct were examined for their ability to form immortalised colonies following serial re-plating in methylcellulose.

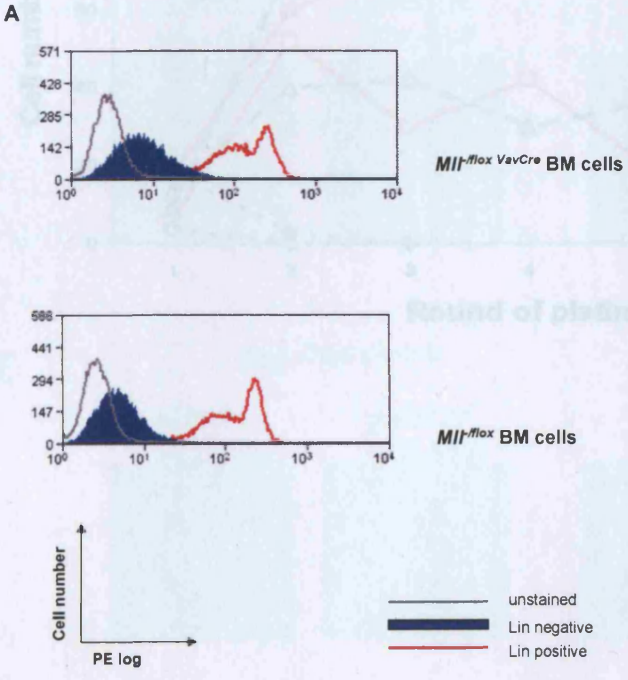
A The genotype of *Mll*$^{-/flox\ VavCre}$ and *Mll*$^{-/flox}$ embryos was confirmed by PCR of DNA extracted from unsorted foetal liver cells (data not shown). These cells were then sorted by MACS for a Lin$^-$ population. This figure shows a representative histogram of the typical efficiency of the isolation.

B The number of colonies formed per 10^4 cells plated following serial re-platings from one representative experiment is shown.

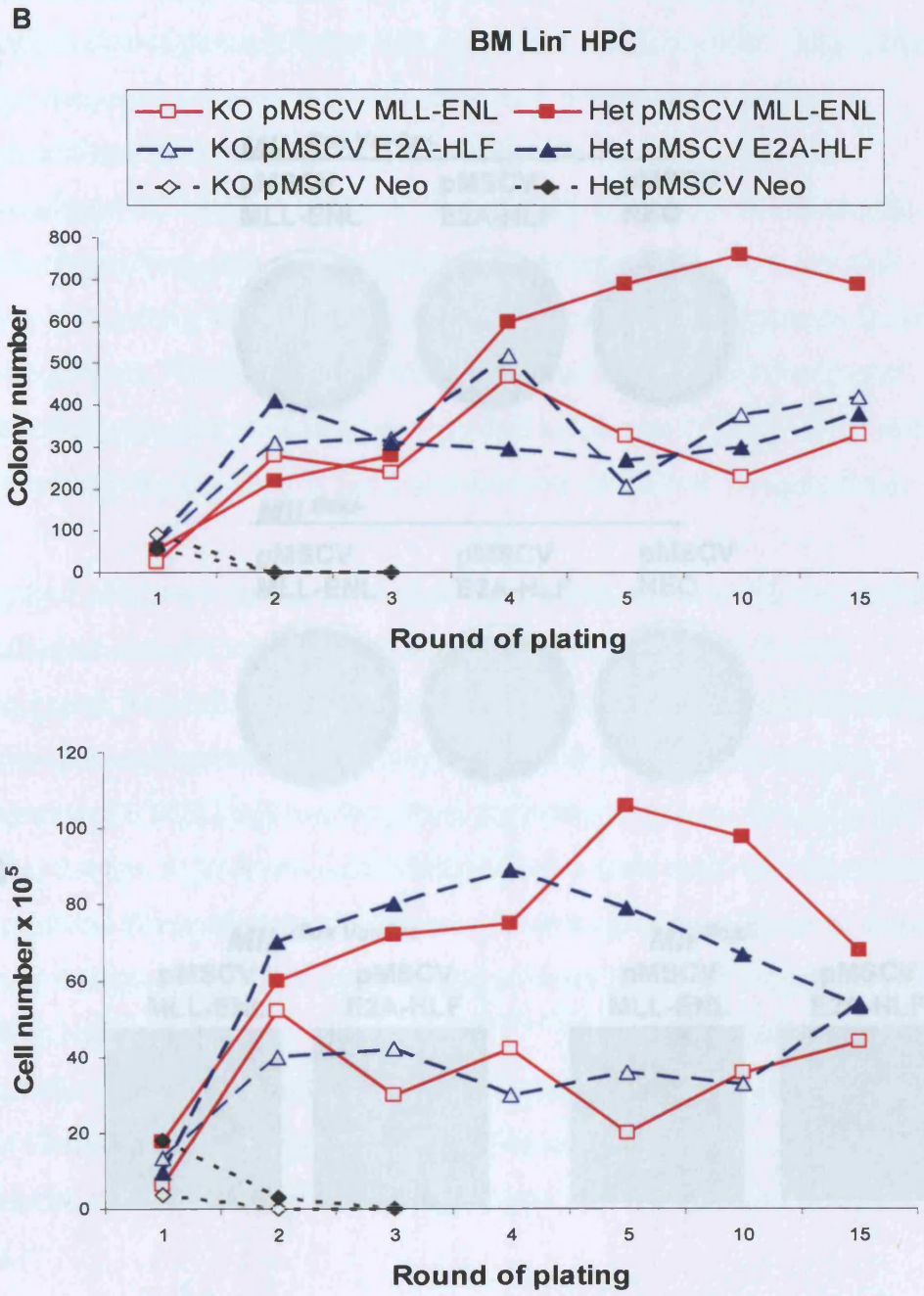
C INT stains of the third round methylcellulose cultures.

D Typical morphology of *Mll*$^{-/flox\ VavCre}$ and *Mll*$^{-/flox}$ MLL-ENL and E2A-HLF colonies (original magnification, x 40).

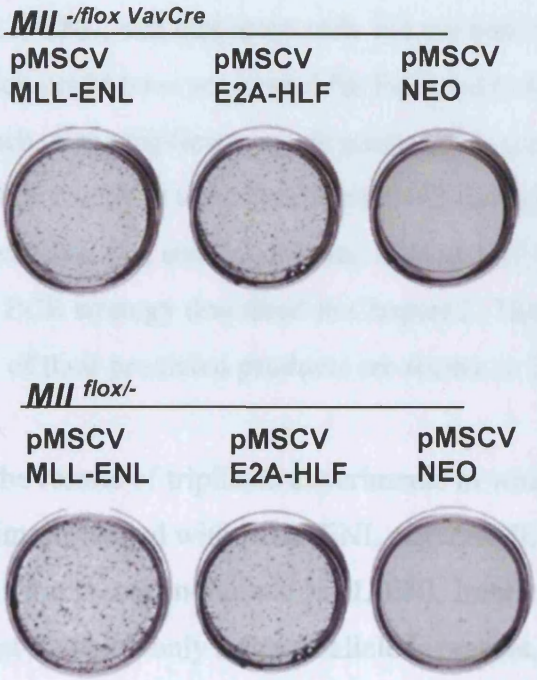
E Typical morphology of the cells from these colonies (original magnification, x 400). Cells were visualised by cytospin preparation followed by May-Grunwald-Giemsa staining.



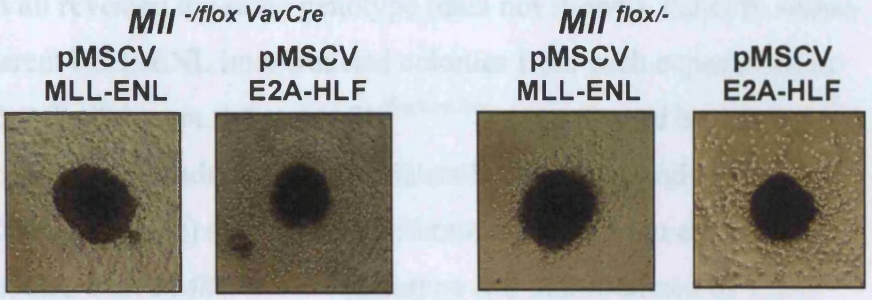
B


BM Lin⁻ HPC
KO pMSCV MLL-ENL
Het pMSCV MLL-ENL
KO pMSCV E2A-HLF
Het pMSCV E2A-HLF
KO pMSCV Neo
Het pMSCV Neo
Colony number
800
700
600
500
400
300
200
100
0
1
2
3
4
5
10
15
Round of plating
Cell number x 10⁵
120
100
80
60
40
20
0
1
2
3
4
5
10
15
Round of plating

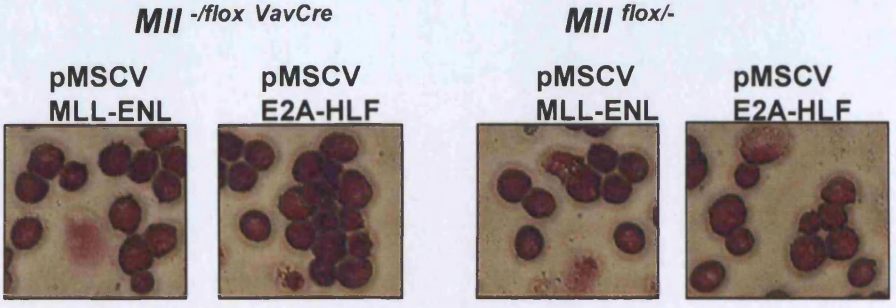
C


Mll -/flox VavCre
pMSCV MLL-ENL
pMSCV E2A-HLF
pMSCV NEO
Mll flox/-
pMSCV MLL-ENL
pMSCV E2A-HLF
pMSCV NEO

D


Mll -/flox VavCre
pMSCV MLL-ENL
pMSCV E2A-HLF
Mll flox/-
pMSCV MLL-ENL
pMSCV E2A-HLF

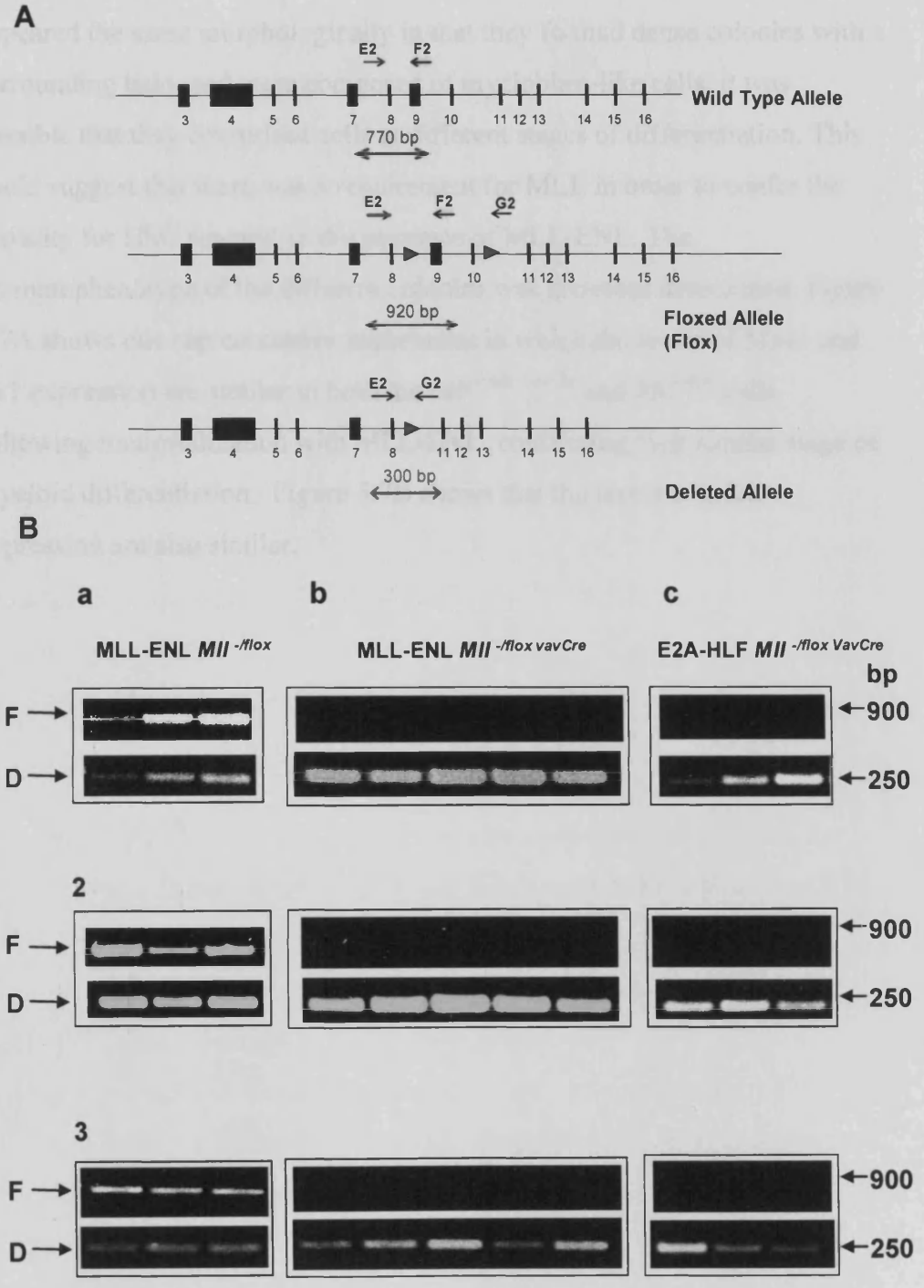
E


Mll -/flox VavCre
pMSCV MLL-ENL
pMSCV E2A-HLF
Mll flox/-
pMSCV MLL-ENL
pMSCV E2A-HLF

Individual colonies were picked from the *Mll*$^{-/flox\ VavCre}$ and *Mll*$^{-/flox}$ methylcellulose plates infected with MLL-ENL and E2A-HLF. These cells were examined to ensure that both *Mll* alleles were absent in cells immortalized by MLL-ENL, and that these cells did not contain non-recombined *Mll* which could have accounted for the results seen. Colonies were picked from each of the triplicate experiments. DNA was extracted from each colony. PCR reactions were then carried out using primers E and F to amplify the Floxed allele (F), and using E and G to amplify the deleted allele (D), using the PCR strategy described in Chapter 2. The position of the primers and the size of their predicted products are shown in Figure 5.6A.

Figure 5.6B shows the results of triplicate experiments in which a series of individual colonies immortalized with MLL-ENL or E2A-HLF were genotyped. Panel a) shows three individual MLL-ENL immortalized colonies from each experiment in which only a floxed allele is present, with the presence of a PCR band resulting from the deleted allele confirming a *Mll*$^{-/Flox}$ genotype. At least seven individual colonies from each experiment were picked and all revealed the same genotype (data not shown). Panel b) shows seven different MLL-ENL immortalised colonies from each experiment in which both *Mll* alleles are deleted (*Mll*$^{-/floxVavCre}$) as confirmed by the presence of the band resulting from the deleted allele only, and absence of the Floxed band. Panel c) shows three different colonies from each experiment also with a *Mll*$^{-/flox\ VavCre}$ genotype and immortalized by E2A-HLF.

Figure 5.6 MLL-ENL immortalises adult HPC lacking both *Mll* loci.
The figure shows the HPC genotype by PCR of DNA extracted from individual colonies immortalised by MLL-ENL from three replicate experiments i, ii and iii.
A shows the targeted *Mll* allele following homologous recombination in ES cells, and the floxed and deleted allele created by recombination at the loxP sites. Black triangles denote the loxP sites. Kp=Kpn1. The primers used to amplify the wild-type allele (primers E and F), and the deleted allele (primers E and G) are also shown. (Figure courtesy of K. McMahon).
B Colonies whose genotype is $Mll^{-/flox}$ (panel a), and $Mll^{-/flox\ VavCre}$ (panel b) are all immortalised by MLL-ENL. WT= wild-type *Mll* band, D= deleted band. Colonies with *Mll* deleted genotype and immortalised by E2A-HLF are shown in panel c.


A
E2
F2
Wild Type Allele
3 4 5 6 7 8 9 10 11 12 13 14 15 16
770 bp
E2
F2
G2
3 4 5 6 7 8 9 10 11 12 13 14 15 16
920 bp
Floxed Allele
(Flox)
E2
G2
3 4 5 6 7 8 11 12 13 14 15 16
300 bp
Deleted Allele
B
a
b
c
MLL-ENL MII -/flox
MLL-ENL MII -/flox vavCre
E2A-HLF MII -/flox VavCre
bp
F
D
900
250
2
3

Although the MLL-ENL immortalized *Mll* $^{-/flox\ VavCre}$ and *Mll* $^{-/flox}$ colonies appeared the same morphologically in that they formed dense colonies with a surrounding halo, and were composed of myeloblast-like cells, it was possible that they comprised cells at different stages of differentiation. This could suggest that there was a requirement for MLL in order to confer the capacity for HSC renewal in the presence of MLL-ENL. The immunophenotype of the different colonies was therefore determined. Figure 5.7A shows one representative experiment in which the levels of Mac1 and Gr1 expression are similar in both the *Mll* $^{-/flox\ VavCre}$ and *Mll* $^{-/flox}$ cells following immortalization with MLL-ENL, confirming their similar stage of myeloid differentiation. Figure 5.7B shows that the levels of c-Kit expression are also similar.

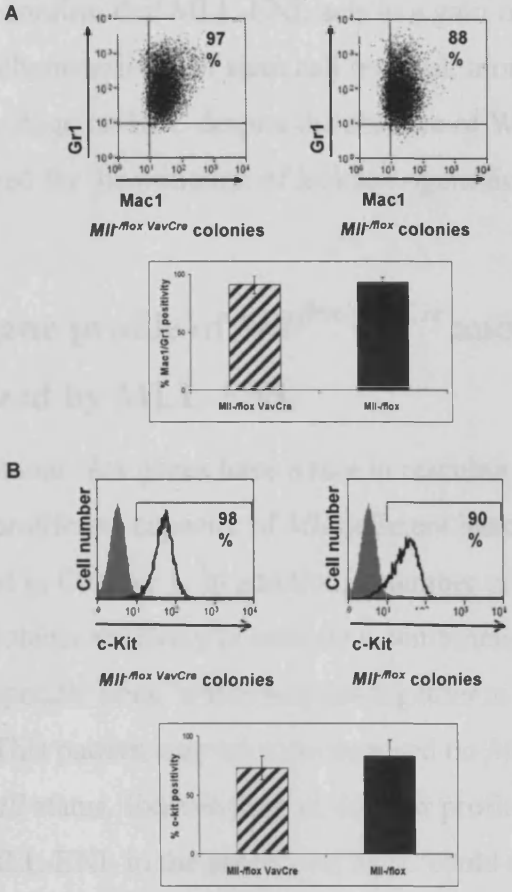


Figure 5.7 *Mll*^{floxVavCre} and *Mll*^{flox} colonies immortalised with MLL-ENL express similar levels of Gr1 and Mac1, and have similar levels of c-Kit expression.

This figure shows the immunophenotype of colonies immortalised with MLL-ENL from knockout and control colonies.

A shows the levels of double positive Mac1 and Gr1 expression in *Mll*$^{-/flox\ VavCre}$ and *Mll*$^{-/flox}$ colonies immortalised with MLL-ENL. A representative experiment of three is shown, together with a plot showing the mean and SD of triplicate experiments.

B Levels of expression of the stem cell marker c-kit are shown in *Mll*$^{-/flox\ VavCre}$ and *Mll*$^{-/flox}$ colonies immortalised with MLL-ENL. The shaded area represents unstained cells, and the bold line represents the sample. A representative experiment of three is shown, together with a plot showing the mean and SD of triplicate experiments

These experiments confirm that MLL-ENL acts in a gain-of-function manner to confer the same characteristics of stem cell renewal, morphology and immunophenotypic stage on HPC despite the absence of WT MLL. MLL is therefore not required for the initiation of leukaemogenesis by MLL-ENL.

5.5 The *Hox* gene profile of *Mll*$^{flox/-\ VavCre}$ and *Mll*$^{-/flox}$ HPC immortalized by MLL-ENL

Studies that suggest that *Hox* genes have a role in rescuing the differentiation ability but not the proliferate capacity of *Mll* deficient haematopoietic cells have been described in Chapter 1. In addition, a number of studies suggest that MLL fusion proteins are likely to activate a combination of specific *Hox* genes, rather than specific ones, which may act together to promote leukaemogenesis. This pattern may be superimposed on *Mll*$^{+/+}$ cells or act independently of *Mll* status. Examination of the *Hox* profile of cells immortalized by MLL-ENL in the absence of MLL could therefore reveal a specific pattern of *Hox* gene expression determined by MLL-ENL.
cDNA was extracted from uninfected *Mll*$^{-/flox\ VavCre}$ haematopoietic cells and from *Mll*$^{-/flox\ VavCre}$ colonies immortalized by MLL-ENL, as well as from uninfected *Mll*$^{-/flox}$ cells and *Mll*$^{-/flox}$ colonies immortalized by MLL-ENL. Q-PCR analysis of *Hoxa, Hoxb* and *Hoxc* genes was conducted in collaboration with the Haematology Department, CCRCB (Queen's University, Belfast). Figure 5.8 shows the genotype of cells from which cDNA was examined and the *Hoxa* profile of immortalized MLL-ENL *Mll*$^{-/flox\ VavCre}$ and MLL-ENL *Mll*$^{-/flox}$ cells.

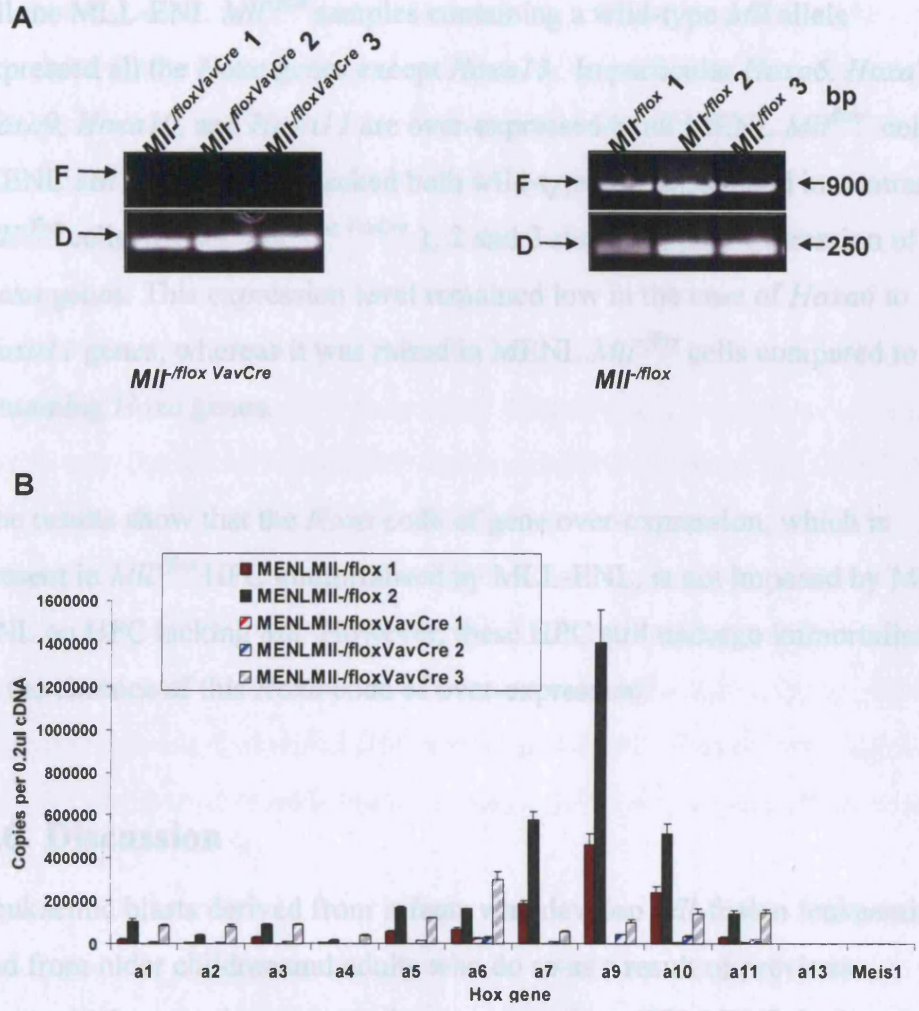


Figure 5.8 The *Hox* gene profile of MLL-ENL *Mll*$^{-/flox\ VavCre}$ compared to MLL-ENL *Mll*$^{-/flox}$ HPC.

A shows the genotype of cells from which cDNA was extracted and analysed by Q-PCR for the profile of *Hox* genes. Colonies were harvested from methylcellulose plates (MLL-ENL *Mll*$^{-/flox\ VavCre}$ and MLL-ENL *Mll*$^{-/flox}$) from three independent experiments (1, 2 and 3), and the genotypes of the cells harvested from each plate confirm that the genotype of the cells harvested are *Mll*$^{-/flox\ VavCre}$ and *Mll*$^{-/flox}$ in each of the triplicate experiments according to the presence of the Floxed (F) and Deleted (D) bands.
B shows the results of Q-PCR analysis of the *Hoxa* profiles of MLL-ENL *Mll*$^{-/flox\ VavCre}$ and MLL-ENL *Mll*$^{-/flox}$ colonies from experiments 1, 2 and 3. The graphs show the number of copies of each *Hoxa* gene per 0.2ul cDNA (25ug of total RNA) for each immortalised cell type.

All the MLL-ENL *Mll*$^{-/flox}$ samples containing a wild-type *Mll* allele expressed all the *Hoxa* genes except *Hoxa13*. In particular *Hoxa6, Hoxa7, Hoxa9, Hoxa10,* and *Hoxa11* are over-expressed in all MENL *Mll*$^{flox/-}$ cells. MENL *Mll*$^{-/flox\ VavCre}$ cells lacked both wild-type *Mll* alleles and in contrast to *Mll*$^{-/flox}$ cells, MENL *Mll*$^{-/flox\ VavCre}$ 1, 2 and 3 show minimal expression of *Hoxa* genes. This expression level remained low in the case of *Hoxa6* to *Hoxa11* genes, whereas it was raised in MENL *Mll*$^{-/flox}$ cells compared to the remaining *Hoxa* genes.

The results show that the *Hoxa* code of gene over-expression, which is present in *Mll*$^{-/flox}$ HPC immortalised by MLL-ENL, is not imposed by MLL-ENL on HPC lacking *Mll.* However, these HPC still undergo immortalisation in the absence of this *Hoxa* code of over-expression.

5.6 Discussion

Leukaemic blasts derived from infants who develop *Mll*-fusion leukaemia, and from older children and adults who do so as a result of previous cytotoxic therapy, demonstrate the presence of one WT *Mll* allele. It would be conceivable that the fusion gene disrupts WT *Mll*, and previous studies suggests that MLL may be recruited by the fusion protein during the leukaemogenesis process (Milne, *et al* 2005b). This study sought to establish whether MLL was required, or redundant, to the process of initiation of leukaemogenesis by *Mll* fusion genes.

The inability of either MLL-ENL or E2A-HLF oncogenes to immortalize *Mll*$^{-/-}$ foetal liver HPC initially suggested that both *Mll* alleles are fundamentally required for self-renewal of HPC. It appeared that self-renewal capacity could not be conferred upon *Mll*$^{-/-}$ cells by a fusion oncogene alone, even when that oncogene had no known requirement for WT *Mll* for its oncogenic function, as is the case with E2A-HLF. However,

subsequent results using adult HPC from a conditional knockout model demonstrate that both fusion genes cause enhanced self-renewal capacity of HPC in which WT *Mll* is absent. This confirms that a typical *Mll* fusion gene such as *MLL-ENL* acts in a gain-of-function manner to initiate leukaemogenesis, rather than doing so by disrupting the WT *MLL* gene.

Further characterization of HPC derived from these $Mll^{-/-}$ foetal liver and from $Mll^{-/flox\ VavCre}$ bone marrow models demonstrated differences in their contribution to the HPC pool (McMahon, *et al* 2007 manuscript submitted) and may explain why foetal $Mll^{-/-}$ HPC failed to immortalize. Foetal livers lacking *Mll* were shown to have a significant reduction in the proportion of LT-HSC and ST-HSC as analysed by cell surface markers, and these $Mll^{-/-}$ cells were unable to reconstitute bone marrow in competitive repopulation assays suggesting that whilst HSC are present in $Mll^{-/-}$ foetal livers they are not able to function as self-renewing stem cells when in competition with normal HSC.

It is therefore possible that the numbers of $Mll^{-/-}$ HSC with self-renewal capacity among the population of isolated $Ter119^{-}$ cells was extremely small compared to that of the $Mll^{+/-}$ HPC, and had a significantly smaller chance of being targeted by the transduced fusion-gene expression constructs. Also, the $Mll^{-/-}$ HSC may have lost their capacity to proliferate and die in culture with cytokines rather than proliferate. These reasons may have resulted in the failure of immortalization of the $Mll^{-/-}$ cells by the fusion genes.

The domains of common fusion proteins that may be responsible for transcriptional de-regulation have been described in Chapter 1. The common fusion partners AF4, AF9 and ENL have been shown to have transactivation properties determined by reporter assays, and may confer these on the fusion protein to cause aberrant transcription of target genes. In addition, a number of fusion partners including ENL have been shown to be directly or

indirectly involved in chromatin remodeling. ENL is homologue of the yeast SWI/SNF subunit, ANC1/TGF3, and is found to be a component of two human SWI/SNF chromatin remodeling complexes (Nie, *et al* 2003). The partner protein appears to assist in accessing chromatin to activate transcription of target genes of MLL-ENL such as the *Hoxa7* promoter. Such mechanisms, in isolation or combined, allow the fusion protein to transactivate its target genes in the absence of WT MLL to cause leukaemia.

The importance of *Hoxa* genes as target genes critical to the initiation of immortalization by MLL fusion proteins was investigated by analysis of HPC immortalized by MLL-ENL but lacking either wild-type *Mll* allele. The results presented show for the first time that the *Hoxa* code of gene over-expression, which is present in $Mll^{-/flox}$ HPC immortalised by MLL-ENL, is not superimposed by MLL-ENL on HPC lacking *Mll.* Critically, these HPC still undergo immortalisation in the absence of this *Hoxa* code of over-expression.

The expression of a *Hox* combinatorial code has been described by a number of studies to be important in the maintenance of the MLL-fusion mediated immortalized phenotype *in vitro* and *in vivo*, with no specific *Hox* gene described as critical to this phenotype (Horton, *et al* 2005, Kumar, *et al* 2004, So, *et al* 2004, Zeisig, *et al* 2004). The *Hoxa* genes, particularly *Hoxa7* and *Hoxa9* have been repeatedly implicated as part of this code. However, all published studies to date use HPC in which at least one wild-type *Mll* allele is present. The *Hoxa* expression profile of these cells may therefore be a result of gain-of-function properties conferred upon MLL by the fusion protein, and may contribute the the maintenance of the immortalized phenotype. However, such altered *Hox* gene expression is not necessarily contributory or essential to the initiation of immortalization by MLL-ENL. MLL is known to be involved in the maintenance, and not the initiation of *Hox* gene expression, and so aberrant MLL function may maintain aberrant

Hox gene expression and so contribute to maintaining the immortalized phenotype. However, the critical genes involved in the initiation of the leukaemic process may be targets of the MLL fusion protein that are distinct from those of normally-functioning MLL, and there may be no initial requirement for genes altered by aberrant MLL function. The data presented here show for the first time that neither wild-type MLL, nor *Hoxa* gene over-expression is required for the initiation of HPC immortalization by MLL-ENL.

6 Conclusions

6.1 A model of leukaemogenesis by gain-of-function of MLL-ENL in primary HSC resulting in aberrant regulation of HTm4 mediated by binding of the target gene via the Menin-binding domain of the fusion protein

I suggest here a model to bring together my observations that MLL-ENL immortalizes HPC in a gain-of-function manner, and that *HTm4* appears to be a target gene of MLL-ENL. MLL-ENL is shown to immortalize HPC without a requirement for WT MLL, showing that alteration of the function of a remaining *Mll* allele by the fusion protein does not contribute to the initiation of immortalization by MLL-ENL. In addition, MLL-ENL does not itself cause aberrant expression of a subset of *Hoxa* genes in the absence of wild-type MLL in immortalized HPC, and these genes are therefore unlikely to be responsible for the initiation of immortalization by MLL-ENL. It is likely, therefore, that MLL-ENL acts in a gain-of-function manner to activate targets distinct from those altered by aberrant MLL function in order to initiate leukaemogenesis. The immortalized phenotype may however be subsequently maintained by aberrant expression of *Hox* genes in the presence of altered expression of a remaining *Mll* gene, as reflected in the results of this and other studies.

The domains of MLL that are replaced by the partner protein to form the fusion protein include the SET domain which, together with other members of the supercomplex, normally contributes to H3-K4 methyltransferase activity to maintain expression of MLL target genes. Transactivation of a set of target genes by the fusion protein uses other mechanisms of

transactivation as described above, such as chromatin remodeling. In comparison to the stability and activity of MLL as part of a chromatin remodeling complex, the MLL fusion protein will not be processed by Taspase 1, and hence will have an altered relationship to the components of a chromatin remodeling complex that will also have different activity to that containing WT MLL. Such a relationship involves the retained ability of the fusion protein to interact with Menin, as opposed to other members of the MLL/HCF complex (Yokoyama, *et al* 2004). The activation of *Hox* targets of MLL fusions and CDKI targets of MLL-AF4 have been shown to rely on such Menin-binding (Milne, *et al* 2005a). Presented here is a model which postulates that MLL-ENL maintains its ability to interact with Menin as part of a chromatin remodeling complex that contributes to the activation of a novel target gene. This novel target, *HTm4*, has been shown to be a highly significantly up-regulated target of MLL-ENL in 32D cells following microarray analysis and has subsequently been validated as a target of MLL-ENL in 32Dcl3 cells. Validation of *HTm4* demonstrates that it is regulated with greatest efficiency in the presence of the Menin binding domain of MLL-ENL. Expression of *HTm4* in HSC is involved in keeping HSC quiescent, and its abnormal activation by MLL-ENL may result in increased cell cycle arrest of cells that may then evade death by chemotherapeutic agents, or acquire further mutations that allow escape from normal cell cycle control and the initiation of a malignant clone.

This model is outlined schematically below.

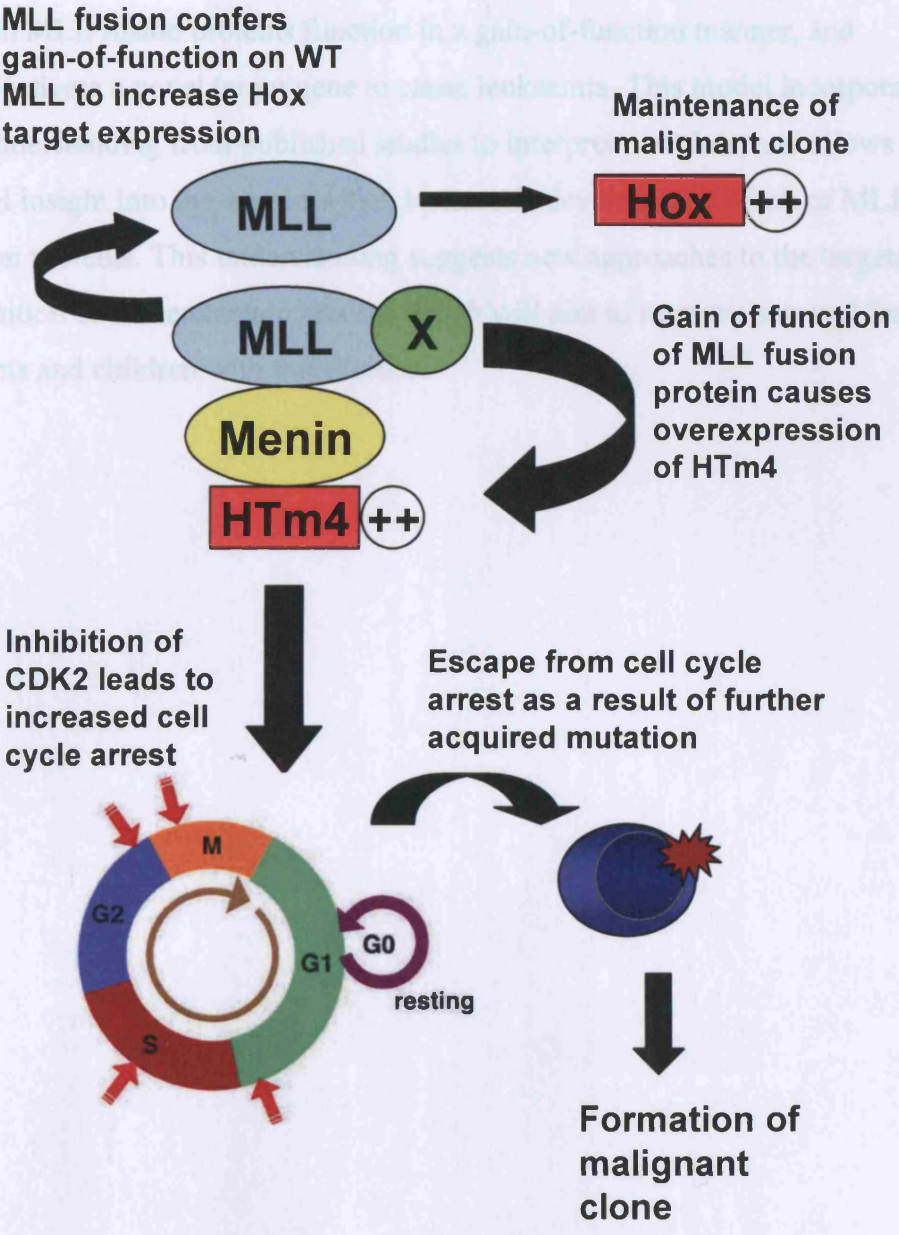


Figure 6.1 A model of leukaemogenesis in which gain-of-function of MLL-ENL in primary HSC results in aberrant regulation of HTm4, which is mediated by binding of the target gene via the Menin-binding domain of the fusion protein.

See text for details.

The data presented in this project support a model of leukaemogenesis in which MLL fusion proteins function in a gain-of-function manner, and transactivate a novel target gene to cause leukaemia. This model incorporates an understanding from published studies to interpret new data, and allows novel insight into the way in which leukaemia develops as a result of MLL fusion proteins. This understanding suggests new approaches to the targeted inhibition of the leukaemic process which will aim to improve survival for infants and children with this disease.

Appendix

Table 10 Classification of highly significant genes down-regulated by MLL-ENL

Regulation of transcription

Probe set ID	Fold change	Gene symbol	Notes
1449333_at	0.7	**Sf3a1**	splicing factor 3a, subunit 1, nuclear mRNA splicing
1418730_at	0.6	**Rnf12**	ring finger protein 12, regulation of transcription, DNA-dependent, protein ubiquitination
1418387_at	0.5	**4930548G07Rik**	chromatin assembly or disassembly, regulation of transcription
1417892_at	0.5	**Sirt3**	sirtuin 3 (silent mating type information regulation 2, homolog) 3 (S. cerevisiae), chromatin silencing, regulation of transcription
1426929_at	0.3	**Brunol4**	bruno-like 4, RNA binding protein (Drosophila), mRNA splice site selection

Regulation of cell cycle

Probe set ID	Fold change	Gene symbol	Notes
1420481_at	0.6	**Cnnm3**	cyclin M3

Regulation of apoptosis

Probe set ID	Fold change	Gene symbol	Notes
1418929_at	0.5	**Esrrbl1**	estrogen-related receptor beta like 1, caspase activation, regulation of apoptosis

Signal transduction

Probe set ID	Fold change	Gene symbol	Notes
1449019_at	0.6	**Akap1**	A kinase (PRKA) anchor protein 1, RNA binding, kinase activity
1424337_at	0.6	**Snx15**	sorting nexin 15, intracellular protein transport, intracellular signaling cascade

Enzymes or transporters

Probe set ID	Fold change	Gene symbol	Notes
1451341_s_at	0.7	**Cebpb**	ubiquitin-conjugating enzyme variant Kua
1421014_a_at	0.5	**Clybl**	citrate lyase beta like
1424746_at	0.5	**Kif1c**	kinesin family member 1C, retrograde transport, Golgi to ER, microtubule-based process
1417865_at	0.4	**Tnfaip1**	tumor necrosis factor, alpha-induced protein 1 (endothelial) potassium ion transport
1423590_at	0.4	**Napsa**	napsin A aspartic peptidase aspartic-type endopeptidase activity, hydrolase activity

Other

Probe set ID	Fold change	Gene symbol	Notes
1425030_at	0.7	**Zfp622**	zinc finger protein 622, RNA binding, zinc ion binding
1426503_a_at	0.6	**Rnf121**	ring finger protein 121
1426835_at	0.6	**Metap1**	methionyl aminopeptidase 1, methionyl aminopeptidase activity
1419471_a_at	0.6	**Nudc**	nuclear distribution gene C homolog (Aspergillus)
1427033_at	0.5	**Dnmbp**	dynamin binding protein
1456772_at	0.4	**Ncf1**	neutrophil cytosolic factor 1, superoxide-generating NADPH oxidase activity, leukotriene metabolism, inflammatory response, cell proliferation
1448241_at	0.2	**Gm2a**	GM2 ganglioside activator protein, sphingolipid metabolism

1452279_at	0.2	**Pfc**	properdin factor, complement activation, alternative pathway
1423739_x_at	0.1	**Aplp2**	amyloid beta (A4) precursor-like protein 2
1419627_s_at	0.1	**Clecsf10**	C-type (calcium dependent, carbohydrate recognition domain) lectin, superfamily member 10

Table 11 Classification of highly significant genes up-regulated by MLL-ENL.

Regulation of transcription

Probe set ID	Fold change	Gene symbol	Notes
1452321_at	6.1	**Wdr9**	WD repeat domain 9, regulation of transcription from Pol II promoter
1436208_at	3.7	**Asb1**	ankyrin repeat and SOCS box-containing protein 1, regulation of transcription, intracellular signaling cascade, male genital morphogenesis
1422855_at	2.1	**Cpsf3**	cleavage and polyadenylation specificity factor 3, mRNA polyadenylylation, mRNA processing

Regulation of cell cycle

Probe set ID	Fold change	Gene symbol	Notes
1454675_at	1.8	**Thra**	thyroid hormone receptor alpha, regulation of cell cycle, cartilage condensation, ossification, regulation, regulation of heart rate, cell growth, organogenesis, negative regulation of transcription

Signal transduction

Probe set ID	Fold change	Gene symbol	Notes
1422188_s_at	19.4	**Tcrgv**	T-cell receptor gamma, variable 4
1420819_at	11.3	**Sla**	*Src*-like adaptor, intracellular signaling cascade
1418892_at	4.0	**Arhj**	*Ras* homolog gene family, member J, cellular morphogenesis, small GTPase mediated signal transduction, regulation of cell shape, actin cytoskeleton organization and biogenesis
1421103_at	3.4	**Bmp2k**	protein amino acid phosphorylation, regulation of bone mineralization, protein kinase activity, ATP binding, transferase activity, phosphatase regulator activity
1450298_at	3.4	**Tnfsf14**	tumor necrosis factor (ligand) superfamily, member 14, cytokine activity, tumor necrosis factor receptor binding
1430640_a_at	3.2	**Prkar2b**	cAMP-dependent protein kinase regulator activity, kinase activity, protein

			amino acid phosphorylation, fatty acid metabolism, signal transduction, cell proliferation, organogenesis
1448548_at	2.5	**Tulp4**	tubby like protein 4, intracellular signaling cascade
1437861_s_at	1.9	**Prkce**	protein kinase C, epsilon, protein amino acid phosphorylation, intracellular signaling cascade chemosensory behavior,regulation of peptidyl-tyrosine phosphorylation
1418594_a_at	1.9	**Ncoa1**	nuclear receptor coactivator 1, chromatin binding, signal transducer activity, receptor activity

Enzymes or transporters

Probe set ID	Fold change	Gene symbol	Notes
1449136_at	448.2	**Epx**	Eosinophils peroxidase
1427052_at	53.5	**Acacb**	Electron transport chain
1448730_at	19.9	**Cpa3**	carboxypeptidase activity, proteolysis and peptidolysis
1420444_at	9.4	**Slc22a3**	solute carrier family 22 (organic cation transporter), member 3
1418989_at	7.9	**Ctse**	cathepsin E, aspartic-type endopeptidase activity, neutrophil collagenase activity
1455045_at	3.5	**serine racemase**	amino acid metabolism, lyase activity, isomerase activity
1454187_at	2.4	**Pkd1l2**	polycystic kidney disease 1 like 2, ion transport

Other

Probe set ID	Fold change	Gene symbol	Notes
1441836_x_at	**473.9**	**Baiap2**	brain-specific angiogenesis inhibitor 1-associated protein 2
1419520_at	**34.9**	**Cml4**	negative regulation of cell adhesion
1459665_at	**16.4**	**Mrvi1**	MRV integration site 1
1437886_at	**6.3**	**Klhl6**	kelch-like 6 (Drosophila)
1455493_at	**3.9**	**Syne1**	synaptic nuclear envelope 1, actin binding, protein binding

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